# PARASITE-DERIVED RESISTANCE

This application is a continuation of application Ser. No. 08/344,616, filed on Nov. 17, 1994, now U.S. Pat. No. 5,580,716, which is a continuation of application Ser. No. 08/068,168, filed on May 28, 1993, now abandoned, which was a continuation of application Ser. No. 07/856,889, filed on Mar. 25, 1992 now U.S. Pat. No. 5,240,841, which was a continuation of application Ser. No. 07/449,049, filed on Dec. 14, 1989, now abandoned, which was a continuation of application Ser. No. 06/842,484, filed on Mar. 21, 1986, now abandoned, which was a continuation-in-part of application Ser. No. 06/714,263, filed on Mar. 21, 1985, now abandoned.

## BACKGROUND OF THE INVENTION

### 1. Field of the Invention

The present invention relates to methods of conferring resistance to parasites, such as viruses, bacteria, and higher parasites, to hosts of the parasite. More particularly, the present invention relates to viral resistance obtained by genetic engineering of the host organism to contain a portion of a replicase enzyme from an RNA virus.

### 2. Description of the Background

A potentially important application of genetic engineering technology is in the area of producing resistance to parasites. The proposals in the prior art that have been systematic and broadly applicable have centered on finding a gene conferring resistance within a strain of the host species or within a related species and transforming the gene into the genome of a susceptible host. This approach may prove effective but has several distinct disadvantages. Resistant forms of the host may not exist or may be very difficult to find for each new race of parasite which arises. Such resistance may be polygenic, making the cloning and transfer of the resistance genes difficult. Where resistance is encoded by a gene, there are commonly already strains of the parasite that have evolved virulence genes for overcoming such host-derived resistances in a gene-for-gene fashion (Flor 1971). Finally, the problem of identifying and isolating the resistance gene from within the large genome of the host will generally remain very difficult. An alternative strategy that addresses these problems is therefore needed.

There have also been proposals for and some work on using genes from organisms unrelated to either host or parasite, which serendipitously have gene products detrimental to a specific parasite. The gene coding for the endotoxin of *Baccillus thuringiensis* (which is toxic to lepidopterous insects) would be an example of this (Held et al., 1982). While this type of approach may prove useful in some specific cases, it clearly represents an opportunistic approach to the problem, as opposed to a systematic methodology that can be applied very broadly.

There already exist some examples of genes, gene derivatives, or gene products of a parasite that can produce a negative interaction with itself or a related genotype. Studies into the susceptibility of plants to infection by viruses have demonstrated that closely related plant viruses or different strains of the same virus will cross-protect a host organism (Hamilton, 1980). In other words, a plant infected by a first virus is often not subject to infection by a second strain of that virus or by a related virus. A similar phenomenon has been observed in animal viruses and has been termed intrinsic interference (Marcus and Carrier, 1967). From the point of view of parasite resistance of the type discussed herein, the key proteins involved in the intrinsic

interference phenomenon are the viral replicase proteins (Marcus and Zuckerbraun, 1970). These same authors proposed that the replicase proteins of the primary infecting virus prevent the replication of the second virus by binding to its replicase attachment sites (Marcus and Zuckerbraun, 1969). A similar proposal has been put forth to explain cross-protection in plants (Gibbs, 1969). In a similar manner, experimenters working with an *E. coli* infected with bacteriaphage 434 have found that infected bacteria are immune to other phages (Lauer et al, 1981; Flashman, 1973; Roberts et al, 1979). Other workers have noticed that endogenous as well as experimentally introduced complementary oligonucleotides can interact with mRNA in a potentially detrimental manner. Simons and coworkers (1983) have suggested that hybridization of a small antisense transcript to *E. coli* Tn10 mRNA contributes to the regulation of transposition of that element. Stephenson and Zamecnik (1978) and Zamecnik and Stephenson (1978) have shown that synthetic oligodeoxynucleotides, complementary to Rous sarcoma virus terminal repeats, diminish normal viral infection and can inhibit viral RNA translation in vitro. However, these discoveries were not applied to the production of host resistance to a parasite.

Despite this fragmentary knowledge in the prior art, there still remains a need for a fully developed technique for producing resistance to parasites that is not based on the traditional methods of using a resistance gene from an immune strain of a host.

## SUMMARY OF THE INVENTION

According, it is an object of this invention to provide a method of conferring resistance to a parasite (specifically an RNA virus) to a host of the parasite which does not rely on the necessity of identifying and isolating a resistance gene from an immune strain of the host.

This and other objects of the invention as will hereinafter become more readily apparent have been accomplished by providing a method for conferring resistance to a parasite to a host of said parasite, which comprises isolating a gene fragment from an RNA virus, wherein the gene from which said gene fragment is derived codes for a replicase enzyme, and inserting said gene fragment or a DNA segment substantially homologous to at least a part of said gene fragment into said host, wherein said gene fragment or DNA segment is expressed as a peptide in said host, wherein said peptide is capable of binding to a replicase binding site in said host.

## BRIEF DESCRIPTION OF THE DRAWINGS

[ The figure shows in schematic form the replicase gene from Qβ and its cleavage sites as described in detail in this application as well as the location of the replicase insert in plasmid pUC18.]

Figure 1 shows in schematic form the replicase gene from Qβ and its cleavage sites as described in detail in this application as well as the location of the replicase insert in plasmid pUC18.

Figure 2 illustrates isolation and assembly of a chimeric gene containing nopaline synthase (NOS) promoter-NPTase II (NPT II) coding sequence-nopaline synthase 3'-nontranslated region. The nopaline synthase promoter was isolated on a 350-bp *Sau*3A fragment that also contained the first 44 bp of the nopaline synthase coding sequence. The sense strand of this fragment was cloned into the *Bam*HI site in M13 mp7 (Messing et al., 1981), the 44 bp was removed by using a modification of a published synthetic primer procedure (Goeddel et al., 1980) with a primer complementary to bases 22-35 of the published nopaline synthase sequence (Depicker et al., 1982), and a 308-bp promoter fragment was obtained after digestion with *Eco*RI. The flush-end of the promoter fragment was joined to a 1-kb *Bgl* II-*Bam*HI fragment carrying the NPTase II coding sequence (a *Bam*HI linker had been inserted at the *Sma* I site) at the filled-in *Bgl* II site (Beck et al., 1982). This fusion regenerates the *Bgl* II site. The chimeric gene was completed by the addition of a 260-bp *Mbo* I fragment that contained the nopaline synthase 3'-nontranslated region. This fragment, which contains a polyadenylation signal (Depicker et al., 1982), was converted to a flush-ended fragment with Klenow polymerase and cloned into the *Sma* I site of a M13 mp8 (Messing et al., 1982) to introduce *Bam*HI and *Eco*RI sites at the 5' and 3' ends, respectively. The resulting 280-bp fragment was joined to the 1,300-bp *Eco*RI-*Bam*HI nopaline synthase promoter-NPTase II coding sequence fragment to generate the complete chimeric gene.

Figure 3 illustrates structures of the pMON120 intermediate vector and chimeric gene introduced into plant cells. Plasmid pMON120 contains the following segments of DNA: the 1.7-kb pBR322*Pvu* II to *Pvu* I fragment that carries the origin of replication and *bom* site (Covarrubias et al., 1981), a 2.2-kb partial

*Cla* I to *Pvu* I fragment of pTiT37 DNA that encodes an intact nopaline synthase (NOS) gene, a 2.7-kb *Cla* I-*Eco*RI fragment of Tn7 (DeGreve et al., 1981) DNA carrying the determinant for spectinomycin/streptomycin resistance, and the 1.6-kb *Hin*dIII-*Bgl* II fragment from the *Hin*dIII-18c fragment of the pTiA6 plasmid. This T-DNA fragment is known to specify two transcripts that are not essential for tumorous growth (Willmitzer et al., 1982; Garfinkel et al., 1981). At the bottom are three chimeric genes inserted at the unique *Eco*RI site of pMON120. The chimeric nopaline synthase-NPTase II-nopaline synthase gene was inserted to give pMON129 and pMON128. In all of these examples, the first plasmid carries the inserted gene as it is drawn in the figure. The second plasmid carries the insert in the opposite orientation to that drawn. Plasmids pMON131 and pMON130 carry a chimeric nopaline synthase-NPTase I-nopaline synthase gene. The final chimeric gene is carried in plasmids pMON140 and pMON139. The bacterial NPTase II promoter and coding sequence have been joined to the nopaline synthase 3'-nontranslated region.

Figures 4A-B illustrate DNA blot hybridization analysis of *in vitro* transformants. Several hundred hormone-independent *in vitro* transformants from each experiment were pooled and total DNA was extracted (Nagao et al., 1981). The DNAs were digested with *Eco*RI and the fragments were separated by electrophoresis and transferred to nitrocellulose (Southern, 1975). (A) Hybridization with NPTase II-specific probe. A gel-purified 3.3-kb *Hin*dIII fragment from Tn*5* (Berg et al., 1975) was used as probe. Lane 1, pMON128::Ti plasmid marker; lane 2, pMON120 transformants; lane 3, pMON139 transformants; lane 4, pMON140 transformants; lane 5, pMON128 transformants; lane 6, pMON129 transformants; lane 7, pMON128 transformants; and lane 8, pMON129 transformants. Lanes 2-6 represent transformants selected for hormone-independent growth prior to scoring for kanamycin resistance; lanes 7 and 8 represent transformants

selected only for kanamycin resistance on medium containing phytohormones. (B) Hybridization with NPTase I-specific probe. A gel-purified 1.2-kb *Ava* II fragment from Tn*601* (Oka et al., 1981) was used as a probe. Lane 1, pMON130::Ti plasmid marker; lane 2, pMON120 transformants; lane 3, pMON130 transformants; lane 4, pMON131 transformants; lane 5, pMON130 transformants; and lane 6, pMON131 transformants. Lanes 2-4 represent transformants selected for hormone-independent growth prior to scoring for kanamycin resistance; lanes 5 and 6 represent transformants selected only for kanamycin resistance on medium containing phytohormones.

Figure 5 illustrates growth of transformants at various antibiotic concentrations. *In vitro* transformants were obtained after cocultivation with *A. tumefaciens* strains carrying cointegrate pMON120, pMON129, or pMON131. Hormone-independent calli (1- to 2-mm diameter) from each experiment were transferred to plates (16 calli per plate) containing the antibiotic concentration shown. After 3 wk, the net growth (wet weight) at each antibiotic concentration was determined and the results were expressed as the % of control growth (growth in the absence of antibiotics). •, pMON129 transformants; Δ, pMON131 transformants; and o, pMON120 transformants.

Figures 6A-E illustrate steps in the SEV system for plant cell transformation. The arrows represent the T-DNA border sequences. *LIH* is a region of homologous DNA for recombination. The tumor genes are represented by *tms* and *tmr* (Garfinkel et al., 1981); *OCS* and *NOS* are octopine and nopaline synthase genes, respectively. The chimeric kanamycin-resistance gene is designated as *kan*$^r$. The bacterial spectinomycin-streptomycin resistance determinant for selection of cointegrates is designated *spc/str*$^r$. Reciprocal recombination of (A) a resident Ti plasmid (pTiB6S3) and (B) pMON120 derivative (pMON128) yields (C) the cointegrate, pTiB6S3::pMON128. After

cocultivation and selection for kanamycin-resistant plant cells either (D) the entire hybrid T-DNA or (E) a truncated T-DNA without tumor genes is transferred into the plant genome.

Figures 7A-B illustrate the analysis of transformed progeny. (A) Leaf callus assay. Surface-sterilized segments of leaves were placed on medium, MS salts (Gibco), B5 vitamins, 3 percent (weight to volume) sucrose, benzyl adenine (1 μg/ml), and naphthalene acetic acid (0.1 μg/ml), *p*H 5.7, containing kanamycin (100 μg/ml). Explants from wild-type plants were unable to grow on this medium, whereas explants from Horsch et al., 1984 transformed plants callused and generated shoots within 3 weeks (data not shown). The explants shown here are from four separate $S_1$ progeny of NPK3. One of the progeny plants is clearly sensitive to kanamycin, whereas the other three are resistant. DNA blot hybridization analysis. (B) Total plant DNA was extracted, purified by CsCl gradient centrifugation, and digested (10 μg) with the restriction enzyme Eco RI as described (Fraley et al., 1983). After transfer of the DNA to nitrocellulose, a nick-translated DNA probe specific for transposon Tn*5* was used to identify a fragment containing the chimeric NOS/NPTII/NOS gene (Fraley et al., 1983). (Lanes a and b) Five-copy (*5c*) and one-copy (*1c*) reconstruction experiments; (lane c) DNA from wild-type (*wt*) control plants; (lane d) DNA from parental NPK3 plant (*Np3*); (lanes e to n) DNA from $S_1$ progeny of NPK3 plant; and (lane o) digested pMON128 plasmid showing the position of the 1.5-kb fragment of the chimeric gene. The letters *r* and *s* denote kanamycin resistance and sensitivity, respectively, in the leaf callus assay.

## DESCRIPTION OF THE PREFERRED EMBODIMENTS

The concept of parasite-derived resistance is that host resistance to a particular parasite can effectively be engineered by introducing a gene, gene fragment, or modified gene or gene fragment of the pathogen into the host. This approach is based upon the fact that in any parasite-host interaction, there are certain parasite-encoded cellular functions (activities) that are essential to the parasite but not to the host. An essential function is one which must operate if the parasite is to survive or reproduce. These functions represent the Achilles heel of the parasite. If one of these functions is disrupted, the parasitic process will be stopped.

"Disruption" refers to any change that diminishes the survival, reproduction, or infectivity of the parasite. Such essential functions, which are under the control of the parasite's genes, can be disrupted by the presence of a corresponding gene product in the host which is (1) dysfunctional, (2) in excess, or (3) appears in the wrong context or at the wrong developmental stage in the parasite's life cycle. If such faulty signals are designed specifically for parasitic cell functions, they will have little effect on the host. Therefore, resistance to a particular pathogen can be achieved by cloning the appropriate parasite gene, if necessary modifying its expression, and transforming it into the host genome. By resistance is meant any reduction in virulence of the parasitic infection or any reduction in the susceptibility of the host to the parasite.

This approach to engineering resistance has important advantages:

1) The source of resistance genes would never be in question, since each parasite would bring with it the genes necessary for deriving resistance.

2) The stability of parasite-derived resistance will generally be greater than the stability of simply inherited forms of host resistance, for reasons that are discussed later in more detail.

3) The difficulties involved in cloning genes from host organisms, which generally have larger genomes relative to their pathogens, are lessened.

4) Parasite-derived resistance will have a minimal effect on the host and should not produce substances harmful to man.

The general concept of parasite-derived resistance is described in U.S. Pat. application Ser. No. 714,263, filed Mar. 21, 1985, which is herein incorporated by reference. The inventors have now specifically reduced to practice a particular embodiment of the invention described generally in the parent application. As will be described in detail later, the specific embodiment is related to the replicase gene of an RNA virus.

All positive-strand RNA viruses that have been investigated contain an enzyme known as RNA replicase, which can utilize viral RNA as the template for the formation of new RNA. RNA replicase is not normally present in a host cell, but is produced when the cell is infected with an RNA virus. viral RNA codes for formation of an RNA replicase which functions only with viral RNA as a template and ignores all other RNA molecules. Accordingly, the RNA replicase represents a viral function that is not part of a normal host function and thereby represents a preferred means of conferring resistance to a host using genes derived from the parasite (viral) organism.

Since replicase enzymes in both plant and animal RNA viruses have striking sequence and functional similarities, the present invention allows the production of resistance to a wide variety of RNA viruses in a simple and straightforward manner. It is possible to use either the specific replicase domain (peptide resulting from a gene fragment as described herein) to confer resistance to other RNA viruses, or equally possible to select from other viruses domains having functional homology for the domain of the Qβ virus used in the current reduction to practice.

The present invention is practiced by isolating a gene fragment from the replicase gene of an RNA virus, preferably Qβ virus, and inserting the gene fragment or a DNA or RNA segment substantially homologous to at least a part of the gene fragment or to a DNA or RNA sequence substantially equivalent to the gene fragment into the host, whereby

the gene fragment or DNA or RNA segment is expressed as a gene product (peptide) in the host. In particular, a gene fragment is selected which produces a peptide containing the binding region (for RNA) of the replicase enzyme. When the gene product, which is less than the entire replicase enzyme and therefore is not capable of functioning as a replicase enzyme, binds to the RNA, it prevents binding of an active replicase enzyme and therefore protects the host against the results of infection by an active virus.

The present reduction to practice was obtained using a gene fragment from the Qβ virus and is therefore a preferred embodiment of the invention. However, gene fragments from the replicase gene of other RNA viruses are equally usable in the method of the present invention.

When a Qβ replicase gene fragment is used, it is preferred to obtain this fragment by cleaving Qβ replicase cDNA with Sau3a at the 5' terminal of the fragment and with NarI at the 3' terminal of the fragment. Use of these designated restriction enzymes produces fragments of the replicase gene of two types, approximately 500 and approximately 1,000 base pairs in length. There are a number of spaced Sau3a cleavage sites in the replicase gene closely spaced from one another so that there are at least five fragments of approximately 1000 base pairs in length and at least three fragments of approximately 500 base pairs in length, all terminating at a NarI cleavage site. The NarI site is beyond the transcriptional termination site of the replicase gene.

It is preferred to prepare a DNA vector by annealing a 5' Sau3a site in the replicase coding region to a BamHI site in the lacZ gene on the plasmid pUC18. This creates a gene, when in-frame, encoding a few amino-terminal amino acids of lacZ followed by a replicase protein domain of varying sizes (depending on the specific Sau3a cleavage sites selected) which terminates with a NarI site beyond the transcriptional termination site of the replicase gene. The production of the replicase domain would then be under the lacZ promoter control. Some of the Sau3a sites will produce an out-of-frame fusion of replicase, but such inoperable gene products are outside the scope of the present invention and can be readily detected since transformation with a vector containing such gene products would not protect the host against infection with an RNA virus.

In addition to the specific vector described above, it is possible to use gene fragments and DNA segments coding for a peptide capable of duplicating the binding function of the replicase enzyme using standard techniques of genetic engineering and other vectors.

In addition to gene fragments obtained from natural sources of RNA replicase enzymes, it is also possible to practice the method of the present invention using a DNA segment encoding a peptide substantially homologous to a peptide encoded by the transcribed portion of the 3' end of an RNA replicase gene. Substantial homology preferably means at least 90% of the amino acids in a given segment are identical (and preferably that any substitutions are conservative substituions), more preferably that no more than two (preferably conservative) amino acids within a given segment are different, and most preferably no more than one (preferably conservative) amino acid within a given segment is different.

It is preferred that a DNA segment coding for such a homologous peptide or for a segment of a natural replicase enzyme contains from 400–1200 base pairs (measured by the 3' end of the transcribed portion of an RNA replicase gene), preferably from 500–1,000 base pairs in length.

As an example of how disease resistance in general can be engineered by the general approach, the discussion below

sets forth in detail how the genes of the bacteriophage Qβ can be used to make *E. coli* resistant to Qβ infection. This example is not to be considered limiting of the invention but is an example of the ease with which the invention can be practiced, now that it is disclosed.

The biology of Qβ and other RNA phages has been extensively documented (Zinder, 1975), and the cDNA sequence of its genome has been determined. The Qβ genome has three major cistrons. These code for a maturation protein (involved in lysis and phage binding to host pili), a coat protein, and a subunit of the replicase enzyme. (A fourth gene product is a minor coat protein which is a read-through product of the coat cistron.)

The life cycle of Qβ is basically as follows. The phage begins by binding to the sex pili of F' *E. coli*, through which it enters the cell and begins to translate its replicase subunit. Its replicase subunit polymerizes with three host subunits normally involved in host protein translation. The resulting hybrid tetrameric enzyme has RNA replicase activity specific for Qβ. This specificity is due to the affinity between the Qβ subunit of the tetrameric replicase and a short segment of the Qβ genome within the replicase cistron. The replicase attaches to Qβ RNA at this binding site and replicates the viral RNA. Late in the life cycle of Qβ, coat protein and maturation protein accumulate in the host. The coat protein then binds to the replicase cistron and thereby represses translation of the replicase subunit. Termination of replication allows viral assembly, and eventually the maturation protein lyses the host, releasing a new population of infective Qβ.

From a conventional (prior art) perspective, the life cycle of Qβ suggests two potential mechanisms for developing resistance. Host-derived resistance might be developed by (1) blocking Qβ binding to sex pili or (2) producing variant host subunits lacking affinity for the Qβ replicase subunit. Blocking Qβ binding is, in fact, a known mechanism for producing Qβ resistance, since non-F' mutants lacking pili are immune to infection (Silverman, Rosenthal, Mobach and Valentine, 1968). However, this strategy clearly disrupts a mechanism which is relevant to the host's fitness as a species. The selection of variant forms of the host subunits which help make up the replicase enzyme may also be a naturally occuring mechanism conferring resistance. Since the host supplies 3 of the 4 subunits of the viral replicase, one might expect mutations within these genes to confer resistance. However, the extent to which these host subunits can be altered is clearly limited, since these subunits are essential to host protein synthesis and the survival of the host. Most of the variants of these host subunits would probably be lethal or sub-lethal for the host. Even non-lethal variants are likely to be suboptimal for protein translation efficiency. Therefore, both of the host-derived resistance mechanisms suggested by the Qβ life cycle would be obtained at the expense of disrupting crucial host functions.

The prospect of being able to transfer genes from parasite to host provides a new approach to resistance. Viewed from this perspective, the life cycle of Qβ suggests at least as many mechanisms of pathogen-derived resistance as host-derived resistance. Several strategies are seen to be promising: (1) deriving resistance from the Qβ coat protein; (2) deriving resistance from a modified Qβ replicase; (3) deriving resistance by cloning the Qβ replicase binding site, and (4) deriving resistance from expression of anti-sense strand RNA sequences. Another strategy involving the maturation protein also appears feasible.

Resistance Derived From the Coat Protein

The Qβ coat protein is known to have a regulatory, as well as a structural role. Late in the phage life cycle, coat protein

binds to and represses the cistron coding for the Qβ replicase subunit, stopping replication and allowing viral assembly (Bernardi and Spahr, 1972). When cDNA to the coat protein translational sequence is linked to an *E. coli* promoter and introduced into *E. coli*, the coat protein is produced in the host. Expression of coat protein (in sufficient quantity) in the host will repress replication of any infecting Qβ, thereby conferring resistance on the transformed host.

Resistance Derived From a Derivative of the Replicase Gene

The Qβ replicase subunit has a dual affinity for a segment of the Qβ genome and the three host replicase subunits (Kamen, 1970; Meyer, Webster and Weissmann, 1981). If the Qβ replicase gene is cloned (as cDNA) and mutagenized, some variant forms will be able to bind to the Qβ replicase site and at the same time fail to polymerize with the host subunits, a requirement to form a functional replicase. Alternatively, a portion of the replicase gene can be cloned to produce a polypeptide containing the functional domain for binding the replicase site but incapable of interacting with the host subunits. A transformed host producing such a modified replicase subunit would be Qβ-resistant if the modified Qβ replicase subunit or a portion of it binds to the replication sites of infecting Qβ and effectively competes with native Qβ replicase for binding sites, thus disrupting Qβ replication. Details of this strategy are set forth in other locations of this specification.

Resistance Derived From Cloned Replicase Binding-Site

The above-mentioned replicase binds to a specific segment of the Qβ genome which is roughly 100 base pairs in length. If this segment is cloned (cDNA) and introduced into the host, it would be transcribed constitutively as mRNA if attached to an appropriate promoter. The transformed host would then be resistant to Qβ because the binding site, which has been shown to compete for binding of the replicase enzyme in vitro (Meyer, Weber and Weissmann, 1976), would limit the free replicase available for Qβ replication.

Anti-Sense Strand Interference

The presence of an RNA complementary to Qβ RNA would allow formation of an RNA—RNA duplex that would block Qβ infection. This can be accomplished, for example, by transcribing a cDNA clone of a portion of Qβ in the reverse orientation in the *E.coli* host. The anti-sense strand RNA produced will then hybridize to the infecting Qβ and interfer with its proper translation or packaging. The advantages of this approach are that potentially any fragment of the viral genome could be used without modification, and it would be extremely difficult for the virus to overcome this form of resistance.

Resistance Derived From Qβ Maturation Protein

Although the maturation protein's mode of action is not yet well understood (Karik and Billeter, 1983; Winter and Gold, 1983), it also represents a potential source of pathogen-derived resistance. A modified maturation protein in the host can block lysis. Alternatively, a repressed operon containing a wild-type maturation gene can be engineered in the host that would be activated by Qβ infection. This would induce premature lysis of a host cell upon initial infection by Qβ, constituting (on the population level) a form of hypersensitivity.

Although the examples set forth above describing methods by which bacteria can be protected from bacteria phage Qβ are related in particular to a specific host/parasite system, the techiques are readily applicable to other systems, such as the protection of other organisms from both viral and non-viral infections. Techniques for achieving these results are set forth in more detail in the following paragraphs.

Virus Resistance

The most likely early application of the concept of parasite-derived resistance is in engineering virus resistance. This is because the viral genome is small, and, since virus only propagates in the host, most of the genome is involved in pathenogenicity. Portions of the viral genome can be cloned and their potential for conferring resistance readily determined. Alternatively, resistance-conferring genes can be discovered empirically by testing the biological effect of various DNA restriction fragments of the viral genome. Most virus-derived resistances are likely to involve a block in replication. The methods described for engineering resistance to Qβ are directly applicable to any virus which a) codes for a protein which helps regulate the virus' reproduction; b) has specific binding sites in its genome; c) synthesizes its own replicase or reverse transcriptase; or d) is bound by complementary reverse strand sequences of nucleic acid. In other words, these methods would apply to essentially all viruses.

While there has been some controversy among biochemists regarding whether plant viruses encode their own replicase, it now seems likely that most plant viruses do code for all or part of their replicases (Hall, Miller and Bujarski, 1982; Dorssers, Van der Meer, Kamen, Zabel, 1983). The first plant virus to have its replication mechanism characterized, turnip yellows mosaic virus, has proven analogous to Qβ (Mouches, Candresse and Bove, 1984). This virus has been shown to to have a hybrid replicase, with its own sub-unit conferring specific binding to its genome. This indicates that the approach described for Qβ replicase would also apply to this virus. It is likely that most or all RNA plant viruses will code either for their own replicase, a subunit of the replicase, or a protein modifying the specificity of the host's RNA polymerase. It is known that there is substantial homology between replicases from a wide variety of RNA variety of RNA viruses (Kamen and Argos, 1984). This means that the replicase-derived resistance strategy outlined for Qβ will be directly applicable to a wide range of plant viruses. Many viruses have not yet been analyzed relative to this genetic structure. However, the very small size of the viral genome and the diversity of potential resistance mechanisms clearly indicates that a viral-derived resistance gene can be derived from any virus simply by using standard shotgun cloning methods and direct screening for subsequent resistance to the virus.

Non-Viral Resistance

The application of parasite-derived resistance to extracellular parasites is more complex than for viral parasites. Since false signals coded for by the host must be recognized by the parasite, parasite-derived resistance will only be useful where mechanisms exist which allow recognition or incorporation by the parasite of non-degraded macromolecules from the host. Van der Plank (1978) has offered persuasive theoretical arguments indicating that such an exchange of macromolecules between the host and the parasite often occurs. There is at least one case where such incorporation has been documented. In the malaria host/parasite system the parasite has been shown to incorporate and utilize a host dismutase enzyme, indicating the presence of a protein exchange mechanism (Fairfield, Meshnick and Eaton, 1983). To the extent that such mechanisms exist in other non-viral host/parasite relationships, the techniques described herein can be applied without significant modification. The existence of protein exchange mechanisms can be determined using monoclonal antibody probes to locate sub-cellular components, in conjunction with 2-D electrophoretic studies searching for host-parasite hybrid proteins.

Given a macromolecular exchange mechanism, a variety of approaches to the engineering of parasite-derived resistance exist for either viral or non-viral parasites. For example, in gene-for-gene host/parasite systems (Flor, 1971; common in viral, fungal, and bacterial pathogens), it is generally found that the parasite's avirulence alleles are dominant to virulence alleles (reviewed in Van der Plank, 1978). This suggests that the avirulence gene products override or block the activity of the virulence gene products-thereby preventing infection. Thus, an avirulence allele cloned from an avirulent strain of the parasite, when introduced and expressed constitutively in a transformed host, can enter the parasite or act at the host-parasite interface and override the infective capacity of an otherwise virulent pathogen. Avirulence alleles can be identified by a variety of methods. For example, in bacteria the virulence-avirulence locus can be cloned by using insertional mutation (employing transposable elements) of the virulent strain and screening for non-virulent mutants or by screening a genomic library for complementation of the virulence allele. The virulence gene can then be introduced into the host to confer resistance. Recently, an avirulence gene has been cloned from the bacterial pathogen *Pseudomonas syringae*. However, the expressed intent of these workers is to clone the resistance gene from the host and the parasite gene has not been introduced into the host in any form (Staskawicz et al 1984). The technique proposed here introduces an entirely new dimension to the classical model of gene-for-gene host/parasite interactions.

Resistance From the Parasite'S Regulatory Genes

A more general strategy for engineering parasite-derived resistance (applicable with or without gene-for-gene interactions) utilizes specific regulatory genes from the parasite. For example, fungal genes regulating haustorial development or sporulation can be introduced into a host, thereby disrupting the normal life cycle of the fungal pathogen, using established techniques of identifying the regulatory protein and searching a genomic library with an antibody probe. Once cloned, such genes can be introduced into a host, where they will disrupt the normal life cycle of the fungal pathogen. This type of regulatory approach appears particularly useful in the engineering of insect resistance. For example, all insects depend on the regulated biosynthesis of juvenile and molting hormones for precise timing of molting, metamorphosis and reproduction. Using the techniques of this invention, it is possible to incorporate into the host genes from the insect pest encoding the activities necessary to produce the insects' hormones, pheromones or neurotransmitters. In the case of neurotransmitters, these polypeptides are typically extremely short (less than 20 amino acids) and are therefore easily sequenced, and artificial genes coding for these sequences can then be synthesized de novo. In the case of non-peptide hormones or pheromones the problem is more difficult but can be overcome. Typically, several enzymatic steps will be required from the starting point of a common precursor in both host and parasite to the biologically active secondary metabolite. This means that several genes in the parasite will have to be identified, cloned and transferred to the host. While this approach does not have the simplicity or directness of other parasite-derived approaches, it is potentially one of the more significant and broad-spectrum applications of parasite-derived resistance, and will generally warrant the time and expense of engineering the latter part of a biosynthetic pathway. The host producing such insect growth regulators or transmitters would be resistant by virtue of disrupting the behavior or life cycle of the insect pathogen, thereby elimi-

nating infection of the primary host. There are examples in nature where plants seemed to have exploited a similar strategy for resistance by evolving genes producing analogs to, or biosynthetic antagonists of, insect hormones (Bowers, 1980).

Another application of parasite-derived resistance is available where an insect or other organism serves as an intermediate host, so that the disease cycle can be disrupted by making the intermediate host resistant to the pathogen, thereby eliminating infection of the primary host. For example, efforts to control malaria have previously focused on eradication of the intermediate host, the Anopheles mosquito. If, however, genes from the Plasmodium pathogen are introduced into the mosquito in a manner to confer resistance by disrupting the life cycle of the parasite, the disease cycle will be broken. This approach is most feasible if the resistance genes is of selective advantage to the intermediate host, allowing resistance genes to be maintained and propagated in natural populations after introduction of modified individuals. This can be done, for example, by concurrently introducing resistance to a pesticide into the intermediate host.

Advantages and Limits of Pathogen-derived Resistance

Parasite-derived resistance represents a systematic and broadly-relevant approach to the problem of how to genetically engineer insect and disease resistance. The rich possibilities of this approach are illustrated by the fact that three different strategies for deriving resistance from the Qβ bacteriophage exist in a parasite having only three genes. There are several distinct advantages of parasite-derived resistance.

One of the most attractive features of parasite-derived resistance is that each new parasite or race of parasite that becomes a problem simultaneously brings with it the specific genes needed to engineer resistance to itself. These genes can be systematically identified within the parasite's genome. Once such genes have been identified, homologous genes in other parasite races or in related parasites will be readily identifiable by DNA hybridization techniques. This eliminates the need for repeated and exhaustive searches through the host's germplasm pools, seeking rare host resistance genes.

Another major advantage of this strategy is that it should not generally be disruptive of host functions. Van der Plank (1978), using evolutionary arguments and population genetics data, has argued that host genes controlling susceptibility exist because they involve essential host functions. Most hosts are genetically susceptible because the susceptible allele is optimal relative to its natural function. Host-derived resistance alleles, therefore, tend to disrupt the optimal functioning of the host. To the extent that this is true, most host-derived resistances attack the pathogen indirectly by replacing an optimal host gene product with a non-optimal host gene product which happens to be incompatible with the parasite. This is seen in the Qβ system, where host-derived resistance is likely to be achieved either by disrupting sex pili formation or by tampering with the host's protein-synthesis machinery. A similar situation exists with sickle-cell anemia, which is harmful to humans when expressed but which confers resistance to malaria in persons who have both a recessive sickle-cell gene and a normal hemoglobin gene. The beauty of the concept of pathogen-derived resistance is that only pathogenic cell functions are attacked and are attacked directly, which will have minimal subsequent effect on the host. The specificity of parasite-derived resistance is not only desirable in terms of being non-disruptive to the host, but also of being non-harmful to

man. Resistance based upon production of general toxicants, such as the natural pesticides of many resistant plant taxa, have been shown to be potentially harmful to man when ingested (Ames, 1983). The specificity of parasite-derived resistance should preclude, to a large extent, any such harm to man.

There are reasons to believe that parasite-derived resistance should be relatively durable compared to host-derived resistance. The ability of parasites to circumvent host-generated general toxicants is well known. Additionally, specific host-derived resistance genes are frequently overcome by matching gene-for-gene mutations to virulence in the parasite (Flor, 1971). In the case of host-derived Q' resistance, alterations in the host replicase sub-units (making them incompatible with the viral subunit, thereby conferring resistance), are easily matched by mutations in the Qβ replicase subunit which restore subunit comparability, constituting a mutation to virulence. However, such gene-for-gene mutations circumventing resistance should be relatively rare in the case of parasite-derived resistance. In this case the parasite would usually be facing a new form of resistance, which it had not previously faced in its evolution. These types of resistances are likely to be very difficult for the parasite to overcome, especially where regulatory genes are involved. For example, if resistance to Qβ was derived from the Qβ coat protein gene, a new virulent Qβ strain could only arise by first having a new binding site develop by mutation in the replicase cistron (without disrupting replicase function) which would not bind the native coat protein. Simultaneously a new coat protein would have to arise by mutation (without disrupting coat protein function) which would bind to the new binding site. Such a simultaneous and complementary set of mutations (which preserved both coat and replicase functions) should be extremely rare.

Last, engineering parasite-derived resistance should be considerably more approachable on the molecular level than engineering host-derived resistance. There are numerous reasons for this: (1) this strategy would generally focus on the molecular biology of relatively simple organisms with short life cycles; (2) it would generally require only the identification and isolation of individual genes from small genomes; (3) unregulated, constitutive expression of the parasite-derived resistance genes would usually be effective; and (4) it would avoid the complex, multigenic biosynthetic pathways which are the likely basis of many existing host-derived resistances.

There do not seem to be any obvious disadvantages to the parasite-derived approach to resistance, except that application of the strategy to non-virus parasites is only possible where mechanisms exist for macromolecular exchange between host and parasite. Most forms of parasitism, especially those forms displaying gene-for-gene resistance, allow ample opportunity for gene-product interactions and will be suitable for engineering parasite-derived resistance.

Techniques for the Production of Resistant Host

As will be readily understood-that those of ordinary skill in the art of genetic engineering, standard techniques of genetic engineering can readily be adopted to attain the goals set forth herein. Protection of a host against a virus, for example, can easily be achieved. Because of the reasons set forth above, it is not necessary to identify the gene being inserted into the host, although identification of the gene will make application of the method easier to perform. In general, genetic information (DNA or RNA) from any virus is isolated using standard procedures and cleaved into pieces of varying lengths, preferably containing at least 20 nucleotides if the DNA is to be transcribed in an anti-sense

direction, or at least a functional portion and preferably an entire gene if the gene is to be expressed. DNA fragments are typically obtained using restriction endonuclease enzymes. The same enzyme (or enzymes) is then used to cleave a vector capable of replicating in the host or inserting into a host's chromosome. The vector can be a natural plasmid or transposon or any part thereof capable of replication in the host and, when desired, production of a gene product from the exogenous parasite gene fragment. Vectors derived from plasmids and other vectors normally present in the host are preferred. The viral DNA is inserted into the vector using standard techniques in either a sense direction (when expression of a gene product is desired) or an anti-sense direction. Proper tailoring of the gene fragment in the vector (e.g., employing appropriate 5' and 3' flanking sequences to ensure regulation, transcription, and translation as desired) is readily achieved using standard techniques, especially when simple constitutive expression is desired, as is suitable in most cases of parasite-derived resistance. As used in this application, the phrase "gene fragment" encompasses both entire genes, DNA segments that contain an entire gene or a portion thereof, and segments of DNA that are incomplete parts of a single gene. The word "gene" encompasses both DNA sequences that code for a peptide gene product and other DNA sequences that form a functional part of a chromosome or plasmid.

Although this specification generally refers to DNA alone when describing genetic information, vectors, or the like, this is done for ease of expression only. Any reference to DNA, unless clearly restricted to DNA and not to RNA, is equally applicable to RNA. For example, pathogenic RNA viruses can be the source of the parasite gene fragment, and non-virulent RNA viruses can act as vectors. In many instances, however, it is easier to work with DNA than RNA (e.g., more DNA restriction endonuclease enzymes are known), and use of cDNA prepared from RNA is a preferred embodiment of the invention when producing resistance to an RNA virus.

After a gene fragment has been isolated, the DNA sequence can be determined and modified, if desired, to produce similar DNA segments capable of being expressed as the same or similar gene products. For example, one or more codons can be replaced by equivalent codons to produce artificial DNA segments coding for the identical gene product. Alternately, a codon can be replaced by a codon that codes for a similar amino acid (e.g., a codon for lucine replaced by a codon for isoleucine or a codon for glutamic acid replaced by a codon for aspartic acid). When used as an antisense strand or binding site, less than 10% non-identical nucleotides are preferred with unmodified gene fragments being most preferred. Greater modification of the gene fragment is possible when a gene product of the parasite gene is being produced. For example, artificial DNA sequences containing a series of codons functionally equivalent (i.e., that code for the same amino acids) to the codon sequence in the parasite gene fragment are considered fully equivalent to the parasite gene fragment since they will produce the same gene product, even though the DNA sequence can be substantially different. Gene products not identical to the natural gene product but retaining the ability to produce a gene product capable of disrupting an essential activity of the parasite can be produced by systematic modification of codons (and thus the expressed gene products) followed by testing for parasite resistance. Such modified DNA segments must be substantially homologous to at least a part of the isolated gene fragment or a DNA sequence functionally equivalent thereto in order to be

considered indicative of parasite-derived resistance. By "substantial homology" is meant at least 80%, preferably at least 90%, and most preferably at least 95% identity between the DNA sequence in question and the sequence to which it is being compared. Identical sequences are also covered by the same phrase. Comparisons for the purpose of determining homology are preferably made over a sequence of at least 15 and more preferably at least 21 nucleotides.

The phrase "isolating a gene fragment", as used in this application, refers to the process of obtaining a gene fragment to be used in the production of resistance in a useful form. The gene fragment does not have to be purified or otherwise separated from other cellular components, although this will occur in many processes. Instead, the word "isolated" is used to indicate that a gene has been obtained in a useful form by a deliberate process. For example, an "isolated gene fragment" can exist in a mixture of fragments from the DNA of a parasite that is to be used in a shotgun cloning procedure. A gene fragment is also still "isolated" when it is present in the form of a recombinant plasmid present in a bacterium being used in a shotgun cloning procedure to identify producers of desired parasite gene products (such as by use of monoclonal antibodies). Likewise, a segment of purified DNA comprising a parasite gene segment and termini from a cloning vector (e.g., obtained by cloning a parasite gene fragment in a bacterial plasmid prior to insertion into the final host) is also encompassed by this term. Other usable forms of gene fragments will be readily apparent to those skilled in genetic engineering.

Insertion of the parasite gene fragment into a host is readily achieved when the host is a bacterium or other unicellular organism since the major advances that have occurred recently in genetic engineering have generally involved insertion of vectors containing exogenous genes into unicellular hosts (especially bacteria and yeasts) and are directly applicable to the present method. "Insertion" encompasses any means of introducing genetic information into a host organism compatible with the limitations discussed in this specification. However, insertion in a manner to provide a heritable characteristic is preferred. In unicellar organisms this can readily be accomplished using heritable plasmids or by insertion of the parasite gene fragment into the host chromosome. These examples are not limiting, and other methods of inserting heritable genetic information, whether into unicellar or higher organisms, are equally applicable to the practice of this invention.

Proven methods for inserting new genes into higher organisms can now be found in a massive volume of current literature. There exist four basic methods of doing this (Baserga, Crose, and Povera, Eds., 1980): (1) direct uptake of DNA or DNA-containing particles by the cell, (2) cell fusion with other cells or ghost cells, (3) microinjection, and (4) infective transformation. A fifth method is being developed which involves the use of accelerated high-velocity one-micron-sized particles for the purpose of carrying DNA into cells and tissues.

Uptake mechanisms include the following: (1) induction of enhanced membrane permeability by use of $Ca^{++}$ and temperature shock (Mandel and Higa, 1970; Dityakin et al., 1972); (2) use of surface binding agents such as PEG (Chang and Cohen, 1979; Krens et al., 1982) or $Ca(PO_4)_2$ (Graham and van der Eb, 1973; Wigler et al., 1979); and (3) phagocytosis of particles such as liposomes (Uchimaya et al., 1982), organelles (Potrykus, 1973), or bacteria (Cocking, 1972), into the cell. These uptake mechanisms generally involve suspensions of single cells, where any existing cell

wall materials have been removed enzymatically. Uptake protocols are generally quite simple and allow treatment of large numbers of cells en masse. In such systems most cells are unaffected, but cell selection procedures are available to recover the rare cells that have been transformed (Powers and Cocking, 1977).

Fusion mechanisms incorporate new genetic material into a cell by allowing it to fuse with another cell. A variation on this theme involves ghost cells. The membrane of killed cells are allowed to fill with a given DNA solution, such that cell fusion incorporates the DNA from the carrier "cell" into the living cell. Cell-to-cell fusion can be induced with the aid of such things as PEG (Bajaj, 1982) and Sendai virus particles (Uchida et al., 1980). As with uptake mechanisms, fusion technologies rely upon the use of single cell suspensions, where cells are enzymatically stripped of any cell wall material. While fusion technologies can have relatively good efficiencies in terms of numbers of cells affected, the problems of cell selection can be more complex, and the resulting cells are typically of elevated ploidy.

Microinjection technologies employ extremely fine, drawn out capillary tubes, which are called microelectrodes. These can be made sufficiently small that they can be used as syringe needles for the direct injection of biological substances into certain types of individual cells (Diacumakos, 1973; Graessmann and Graessmann, 1983). One modification of microinjection involves pricking with a solid-glass drawn needle, which carries in biological solutions which are bathing the cell (Yamomoto et al., 1981). Another modification is called ionophoresis (Purres, 1981; Ocho et al, 1981), which uses electrophoesis of substances out of the microelectrode and into the cell as an alternative to high pressure bulk flow. Microinjection procedures can give extremely high efficiencies relative to delivery into the cell. Because of this, microinjection has been used successfully in the transformation of individual egg cells.

In another example, foreign DNA was successfully injected into cotton pollen tubes without the pollen being damaged or its germination being inhibited. Although this involved a resistance gene from another plant instead of a parasite gene, the same technique can be used in the practice of the present invention. DNA was injected into the nucleus of cotton pollen grains germinating on cellophane using micro-manipulators and a micro-injection system. This operation was carried out on the fixed stage of an inverted research microscope equipped with Nomarski differential interference optics. Foreign DNA in a recipient nucleus was detected by epifluorescence after the incorporation of a fluorescent marker in the injected material. The DNA was introduced using "quickfill" tubing drawn to a tip diameter of 0.5 micron, and the DNA was injected into the nucleus iontophoretically. The germinating pollen was returned to the style where it continued to grow and fertilize the ovule. About 20 injections can be carried out per day. Seeds from the micro-injected plants were planted, and seedlings were raised and screened. Screening may be carried out by testing for the presence of the foreign gene by Southern blotting or for the presence of the gene product by means of enzyme inhibition assays. In addition, screening for insect resistance of the developing square and boll can be utilized when cotton is the host. Other plants can be treated in the same manner.

Infective transformation employs non-injurious infective agents of the host, such as viruses, which naturally transmit part of their genome into the host. In plants, the principal mode of transformation now being practiced is the use of the

[infective agent *Agrobacterium tumefaciens*. This bacterium will naturally colonize cells of any dicotyledonous plant and transmit a specific "T-region" of its Ti-plasmid into the plant chromosome. Other plant vectors useful for the transformation of plants can similarly be used. Genes of interest can now be routinely engineered into the T-region and can be transmitted to the plant by the bacterium (see Fraley et al., 1983). Simple conincubation (growing plant cells and bacterial cells together) has been shown to be extremely effective in transforming plant protoplasts and leaf disks, and whole transformed plants have now been regenerated in numerous plant species (see Horsch et al., 1984). In mammals, naturally infective retroviruses have been used to construct naturally transforming vectors which insert engineered DNA into the mammalian chromosome, in a manner similar to *Agrobacterium tumefaciens*. This transformation mechanism is considered extremely promising for animal and human gene therapy (see Anderson, 1984). ]

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Genes of interest can now be routinely engineered into the T-region and can be transmitted to the plant by the bacterium (see Fraley et al., 1983). Fraley et al, 1983, states as follows:

Chimeric bacterial genes conferring resistance to aminoglycoside antibiotics have been inserted into the *Agrobacterium tumefaciens* tumor-inducing (Ti) plasmid and introduced into plant cells by *in vitro* transformation techniques. The chimeric genes contain the nopaline synthase 5' and 3' regulatory regions joined to the genes for neomycin phosphotransferase type I or type II. The chimeric genes were cloned into an intermediate vector, pMON120, and inserted into pTiB6S3 by recombination and then introduced into petunia and tobacco cells by cocultivating *A. tumefaciens* cells with protoplast-derived cells. Southern hybridization was used to confirm the presence of the chimeric genes in the transformed plant tissues. Expression of the chimeric genes was determined by the ability of the transformed cells to proliferate on medium containing normally inhibitory levels of kanamycin (50 μg/ml) or other aminoglycoside antibiotics. Plant cells transformed by wild-type pTiB6S3 or derivatives carrying the bacterial neomycin phosphotransferase genes with their own promoters failed to grow under these conditions. The significance of these results for plant genetic engineering is discussed.

Abbreviations: bp, base pair(s); kb, kilobase(s); NPTase I and NPTase II, neomycin phosphotransferase, types I and II, respectively; Ti plasmid, tumor-inducing plasmid; T-DNA, transferred DNA; Ri plasmid, root-inducing plasmid.

The transformation of plant cells by virulent strains of *Agrobacterium tumefaciens* has been studied extensively by several laboratories (Chilton et al., 1977; Van Larebeke et al., 1974; Kerr et al., 1977; Braun, 1956). A small fragment of the tumor-inducing (Ti) plasmid, called transferred DNA (T-DNA), is known to be transferred to and stably incorporated in the nuclear DNA of transformed plant cells (Chilton et al., 1980; Yadav et al., 1980; Willmitzer et al., 1980). The T-DNA is actively transcribed in plant cells (Willmitzer et al., 1982; Bevan et al., 1982; Gelvin et al., 1981) and specific gene products have been shown to be responsible for the observed phytohormone-independent growth characteristics (Leemans et al., 1982; Garfinkel et al., 1981) and novel metabolic capacities (Holsters et al., 1980) exhibited by crown gall tumor cells. The transfer and insertion of T-DNA into plant DNA is thought to involve repeated nucleotide sequences present near the T-DNA "borders" (Zambryski et al., 1982; Yadav et al., 1982) as well as other genes of unknown function located in specific virulence regions outside of T-DNA (Hille et al., 1982; Klee et al., 1982).

In spite of considerable understanding of the *A. tumefaciens*-Ti plasmid system, several problems remain which limit its use as a vector for genetically modifying higher plants. Because of the high levels of phytohormones produced by crown gall tumor cells (Akiyoski et al., 1983) they have generally proven recalcitrant to attempts to induce regeneration into whole plants (Braun et al., 1976; Yang et al., 1980). Exceptions to this are cases in which, as a result of aberrant integration or spontaneous deletion events, transformed cells have lost all or part of the Ti plasmid tumor genes and can now be regenerated (Otten et al., 1981; Wullems et al., 1981a). In addition, transformation of cells by weakly virulent, mutant Ti plasmids (Barton et al., 1983) and transformation by root-inducing (Ri) plasmids (Chilton et al., 1982; White et al., 1982) have been shown to produce callus that can be regenerated into whole plants. However, these plants often display morphological aberrations and may retain certain tumorous properties (Spano et al., 1982). Another obstacle has been the failure to obtain expression from a variety of foreign genes that

have been introduced into plants (Barton et al., 1983; Chilton et al., 1981). Reasons for this include the fact that, up to now, most studies have utilized either heterologous genes from bacteria, fungi, and mammalian cells whose regulatory regions may not be recognized by the plant RNA polymerases or highly regulated plant genes which are normally expressed in specialized tissues and which may not be transcribed in undifferentiated crown gall tumor tissue.

To bypass the dependence on tumor genes for identifying transformed plant cells and to overcome the barriers to gene expression in plants, chimeric genes that function as dominant selectable markers have been assembled. These contain the neomycin phosphotransferase (NPTase) coding sequences from the bacterial transposons Tn*5* (type II) or Tn*601* (type I) joined to the 5' and 3' regulatory regions of the nopaline synthase gene from the Ti plasmid. Fraley et al., 1983, describes the construction of these chimeric genes and their introduction and expression in plant cells.

DNA Preparation. Plasmid pBR322 and its derivatives or M13 replicative form DNAs were purified by using either a Triton-X-100/CsCl procedure (Davies et al., 1980) or a large-scale alkaline lysis procedure (Ish-Horowicz et al., 1981), followed by purification on hydroxylapatite (Colman et al., 1978).

DNA fragments were isolated by electroelution into dialysis bags after polyacrylamide gel electrophoresis and band excision or by adsorption onto NA-45 DEAE membrane (Schleicher & Schuell) after agarose gel electrophoresis (Maniatis et al., *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratories, Cold Spring Harbor, N.Y., p. 504 (1982)).

The *Bam*HI synthetic DNA linkers (5' C-C-G-G-A-T-C-C-G-G, SEQ. ID. No. 1) were purchased from Collaborative Research (Waltham, MA). Other synthetic DNAs were synthesized by using a modification of the phosphite procedure (Adams et al., 1983).

Enzymes. All restriction endonucleases and the large Klenow fragment of DNA polymerase I were obtained from New England Biolabs or Bethesda Research Laboratories and were used according to the instructions of the supplier. Phage T4 DNA ligase was prepared as in Murray et al., 1979. DNA fragment assembly

was carried out as described (Maniatis et al., *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratories, Cold Spring Harbor, N.Y., p. 504 (1982)).

Transformation of *Escherichia coli* cells. Plasmid DNAs were introduced into *E. coli* cells by using $CaCl_2$-treated or $RuCl_2$-treated cells (Maniatis et al., *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratories, Cold Spring Harbor, N.Y., p. 504 (1982)). The recipient *E. coli* K-12 strains were SR200 = C600 *thr pro recA56 hsdR($r^-m^+$)* (Rogers et al., 1980); LE392 = ED8554 *hsdR($r^-m^+$)* (Maniatis et al., *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratories, Cold Spring Harbor, N.Y., p. 504 (1982)); SR20 = GM42 = *his dam-3* (Bale et al., 1979); and the M13 phage host, JM101 (Messing et al., 1981). Cells carrying recombinant plasmids were selected or grown (or both) on Luria medium plates or broth at 37°C containing appropriate antibiotics (ampicillin, 200 μg/ml; spectinomycin, 50 μg/ml; and kanamycin, 40 μg/ml).

Introduction of pMON120 Derivatives into *A. tumefaciens*. Plasmid pMON120 or its derivatives were transferred to a chloramphenicol-resistant *A. tumefaciens* strain GV3111 = C58C1 $Cm^R$ carrying pTiB6S3tra$^c$ (DeGreve et al., 1981) by using a triparental plate mating procedure (Ditta et al., 1980). Briefly, 0.2 ml of a fresh overnight culture of LE392 carrying pMON120 or its derivative was mixed with 0.2 ml of an overnight culture of HB101 (Maniatis et al., *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratories, Cold Spring Harbor, N.Y., p. 504 (1982)) carrying the pRK2013 (Ditta et al., 1980) plasmid and 0.2 ml of an overnight culture of GV3111 cells. The mixture of cells was spread on an LB plate and incubated for 16-24 hr at 30°C to allow plasmid transfer and recombination. The cells were resuspended in 3 ml of 10 mM $MgSO_4$ and a 0.2-ml aliquot was then spread on an LB plate containing 25 μg of chloramphenicol per ml and 100 μg each of spectinomycin and streptomycin per ml to select *A. tumefaciens* carrying the pMON120 derivatives. After incubation for 48 hr at 30°C, ≈10 colonies per plate were obtained. Control matings between HB101/pRK2013 cells and GV3111 cells never gave rise to colonies after this selection. Typically, one

colony was chosen and grown at 30°C in LB medium containing chloramphenicol, spectinomycin, and streptomycin at the same concentrations given above.

Protoplast Isolation and Culture. "Mitchell" petunia plants were grown in environmental chambers under fluorescent and incandescent illumination (≈5,000 lux, 12 hr/day) at 21°C in a 50:50 mixture of vermiculite and Pro-mix BX (Premier Brands, Quebec, PQ, Canada). Leaves were surface sterilized, cut into 2-mm strips, and enzymatically digested as described (Ausubel et al., 1980). The resulting protoplasts were purified by passage through stainless steel meshes and by density floatation as described (Ausubel et al., 1980). The protoplasts were plated in tissue culture flasks (T75, Falcon; 6 ml per flask) at a cell density of $10^5$ cells per ml in culture medium, MS salts (GIBCO), B-5 vitamins, 3% (wt/vol) sucrose, 9% (wt/vol) mannitol, 1 μg of 2,4-D per ml, and 0.5 μg of benzyl adenine per ml, pH 5.7.

Cocultivation of *A. tumefaciens* Cells with Plant Protoplasts. On day 2 after protoplast isolation, aliquots (10-50 μl) of an overnight culture of *A. tumefaciens* cells were added to each flask (final bacterial cell density = $10^8$ cells per ml) and cocultivation with plant cells was carried out for 24-30 hr essentially as described (Wullems et al., 1981b). On day 3, 6 ml of culture medium (lacking phytohormones and mannitol) containing carbenicillin (1.5 mg/ml) was added to each flask (final concentration = 500 μg/ml) to prevent further bacterial growth. On day 4, an additional 6 ml of the above medium (containing carbenicillin at 500 μg/ml) was added. On day 6, 0.5 ml of the cell mixture was transferred to and spread in a thin layer on the surface of double-filter feeder plates (Horsch et al., 1980). These consisted of agar medium (MS salts, B-5 vitamins, 3% sucrose, 3% mannitol, 0.1 μg of indole acetic acid per ml, and 500 μg of carbenicillin per ml at pH 5.7), a layer of *Nicotinia tabacum* suspension cells, a tight fitting 8.5-cm Whatman filter paper disc (guard disc), and a 7.0-cm Whatman filter paper disc (transfer disc). After 7-10 days, microcolonies (≈0.5 mm) were observable on the feeder plates and the transfer disc was removed and placed on selection medium (MS salts, B-5 vitamins, 3% sucrose, 500 μg

of carbenicillin per ml at pH 5.7) lacking phytohormones. Within 2 wk, hormone-independent transformants could be readily distinguished as green colonies against a background of dying, brown nontransformed cells. The transformation frequency in these experiments was $\approx 10^{-1}$. The hormone-independent transformants were then transferred to medium (MS salts, B-5 vitamins, 3% sucrose, 500 μg of carbenicillin per ml at pH 7.5) containing kanamycin (50 μg/ml).

Analysis of Transformants. Octopine and nopaline synthase activities were determined as in Otten et al., 1978, with the substitution of [$^{14}$C]arginine (Amersham, 0.5 μCi/2.5-μl assay; 1 Ci = 3.7 x $10^{10}$ Bq) for the unlabeled arginine in the assay buffer. The conditions for electrophoresis were as described (Otten et al., 1978) and the resulting electrophoretograms were exposed to x-ray film (Kodak, XAR-5) for 16-24 hr. The positions of octopine, nopaline, and arginine were established by their comigration with authentic standards.

Callus for NPTase-assays were frozen in liquid $N_2$ and extracted by using a mortar and pestle in a minimal volume of buffer (0.2 M Tris-HCl/2 mM EDTA/7.5% polyvinylpolypyrolidone). The crude extract was clarified by centrifugation (Eppendorf; Brinkmann) and assays were performed as described (Jimenez et al., 1980).

NPTase coding sequences were used in the initial chimeric gene constructions described in this study because plant cells were determined to be sensitive to various aminoglycoside antibiotics (unpublished data), and the expression of NPTase in yeast (Jimenez et al., 1980) and mammalian cells (Colbere-Garapin et al., 1981; Southern et al., 1982) has been previously shown to confer resistance to the antibiotic, G418. The nopaline synthase gene promoter and 3'-nontranslated regions were selected because this gene has been well characterized (Bevan et al., 1982; Depicker et al., 1982) and it is known to be expressed constitutively in most plant tissues transformed with the *A. tumefaciens* Ti plasmid (Tempé et al., 1982).

Construction of Chimeric Genes. The nopaline synthase promoter region, obtained on a 350-base-pair (bp) *Sau*3A fragment

from the *Hin*dIII-23 fragment of pTiT37 (Fig. 2; Depicker et al., 1982), was engineered to remove the entire nopaline synthase coding sequence. The resulting promoter fragment that extends from base -264 to base 35 of the nopaline synthase sequence (Depicker et al., 1982) was positioned next to the *Bgl* II site located just outside the NPTase II coding sequence (Beck et al., 1982). In addition, a 260-bp *Mbo* I fragment, extending from base 1,297 to base 1,554 of the published nopaline synthase sequence (Depicker et al., 1982), was isolated from the *Hin*dIII-23 fragment. This *Mbo* I fragment contains the nopaline synthase 3'-nontranslated region and polyadenylation site. This fragment was ligated together with the *Eco*RI-*Bam*HI fragment that contained the nopaline synthase promoter and NPTase II structural gene to yield the intact chimeric gene on a 1.5-kilobase (kb) *Eco*RI fragment (Fig. 2). A second chimeric gene, containing the nopaline synthase promoter and 3'-nontranslated region joined to the NPTase I coding sequence (Fig. 3), was constructed in a similar fashion. As controls, plasmids were constructed that contained an intact NPTase II promoter and structural sequence with the nopaline synthase 3'-nontranslated region (pMON139 and pMON140; Fig. 3).

Introduction of Chimeric Genes into the Ti Plasmid. The vector pMON120 used for the transfer of the chimeric genes into *A. tumefaciens* cells is shown in Fig. 3. Its essential features include (*i*) a segment of pBR322 DNA for replication in *E. coli*, (*ii*) a segment from pTiT37 that contains a functional nopaline synthase gene to facilitate the rapid identification of transformants, (*iii*) a segment of Tn7 carrying the spectinomycin/streptomycin-resistance determinant for selection in *A. tumefaciens*, (*iv*) a DNA segment obtained from the pTiA6 T-DNA fragment *Hin*dIII-18c (see T-DNA map, Leemans et al., 1982), which is included to provide homology for recombination with a resident octopine-type Ti plasmid in *A. tumefaciens*, and (*v*) unique restriction sites (*Eco*RI and *Hin*dIII) for insertion of the chimeric genes. The pMON120 plasmid and derivatives were introduced into *A. tumefaciens* as described above.

Selection of Kanamycin-Resistant Petunia Transformants. Several hundred hormone-independent calli (1-2 mm in diameter) obtained from cocultivation experiments with *A. tumefaciens* strains carrying pTiB6S3::pMON120 (or derivatives) recombinant plasmids were pooled and analyzed by DNA blot hybridization for the presence of the chimeric genes (Fig. 4). The results confirm the presence of the expected 1.6-kb *Eco*RI fragment, which carries the chimeric nopaline synthase-NPTase II-nopaline synthase gene in pMON128 and pMON129 transformants, and the control NPTase II-NPTase II-nopaline synthase construct in pMON139 and pMON140 transformants (Fig. 4A).

Similar results were obtained for pMON130 and pMON131 transformants, which contain the chimeric nopaline synthase-NPTase I-nopaline synthase gene on a 1.5-kb *Eco*RI fragment (Fig. 4B). No hybridization with either the Tn*5*- or Tn*601*-specific probe was detected in transformants containing only the pMON120 vector. Other minor brands of hybridization are present in the pMON129 and pMON140 transformants; these may be attributable to partial digestion or aberrant integration events and their assignment awaits further analysis of clonal tissue. Blot hybridization analysis of DNA from these transformants using T-DNA-specific probes confirmed the presence of the expected internal T-DNA fragments in the transformed tissues and ruled out any possibility that the plant tissue was contaminated by *A. tumefaciens* cells (data not shown).

Other transformed, hormone-independent calli from these experiments were transferred to agar medium containing kanamycin (50 μg/ml) and these were scored after 2-3 wk for resistance to the antibiotic. All transformants obtained from experiments utilizing pMON120, pMON139, or pMON140 failed to grow on medium supplemented with kanamycin, whereas all the transformants from experiments utilizing pMON128, pMON129, pMON130, or pMON131 grew on medium containing the antibiotic at rates comparable to growth on normal medium. A quantitative assessment of the level of resistance conferred by the chimeric genes is shown for pMON120, pMON129, and pMON131 (Fig. 5). The results are based on the net growth of

independent transformants on medium containing the levels of antibiotic shown in the figure, compared to growth in the absence of antibiotics. It is apparent that transformants containing the chimeric nopaline synthase-NPTase II-nopaline synthase gene (pMON129) require ≈20-fold higher levels of kanamycin to depress net growth by 50% in comparison to transformants lacking the chimeric gene (pMON120). Similar results were obtained for pMON128, which contains the chimeric gene in the opposite orientation in the pMON120 vector (not shown). Transformants containing pMON139 and pMON140 have dose responses identical to pMON120. Transformants containing pMON130 or pMON131 (chimeric nopaline synthase-NPTase I-nopaline synthase gene) are less resistant to kanamycin than those containing pMON128 or pMON129 (results shown for pMON130). However, this level of resistance (≈3-fold greater than control cells) is still quite adequate for selection (see below).

Additional cocultivation experiments were carried out without hormone-independent selection (i.e., medium supplemented with phytohormones which support the growth of nontransformed cells). The resulting microcolonies (≈1 mm) were transferred to phytohormone-supplemented medium containing kanamycin (50 μg/ml) and within 2-3 wk, growing colonies were readily observable on plates containing cells that were transformed with pMON128, pMON129, pMON130, or pMON131. The frequency of transformation obtained by using antibiotic selection was comparable to that obtained by using hormone-independent selection. Opine (data not shown) and Southern hybridization analysis (Fig. 4A, lanes 7 and 8; Fig. 4B, lanes 5 and 6) of the kanamycin-resistant colonies confirmed that they were indeed transformants. No growing colonies were observable on plates containing cells transformed by pMON120, pMON139, or pMON140 plasmids.

The expression of the prokaryotic NPTase I and NPTase II enzymes in plant cells by using the intermediate vector pMON120 probably depends on transcription from the nopaline synthase promoter. Support for this comes from the facts that (*i*) the prokaryotic genes with their own promoters do not confer

antibiotic resistance to petunia cells (Figs. 3 and 5) and (*ii*) all of the constructions function identically in either orientation in the pMON120 vector, suggesting that transcription does not initiate elsewhere in the vector. RNA blot hybridization experiments have confirmed the presence of NPTase II-specific mRNA in the transformed tissues and nuclease S1 mapping experiments demonstrate the expected 5' and 3' ends for the chimeric NPTase II mRNA (data not shown). In addition, low levels of neomycin-dependent NPTase II activity have been reproducibly observed in crude cell extracts from tissues transformed with pMON128 or pMON129 (no activity has been detected in extracts from control cells or cells transformed with pMON120, pMON139, or pMON140).

The useful range of these chimeric antibiotic resistance genes appears to be quite broad. In addition to the results presented for petunia, successful selection of aminoglycoside-resistant transformants has also been demonstrated for tobacco, sunflower, and carrot (results not shown). It seems likely that most plants within the host range of *A. tumefaciens* could be transformed and identified in this manner. Those plant cells that are not particularly sensitive to kanamycin may be killed by other aminoglycoside antibiotics. In this respect pMON128 (or pMON129) and pMON130 (or pMON131) also function to confer resistance to G418 and neomycin on petunia, carrot, sunflower, and tobacco (unpublished data).

The availability of dominant selectable markers on small plasmids such as pMON120 should facilitate the development of alternate, non-*A. tumefaciens*-mediated methods for transforming plant cells such as spheroplast fusion (Hasezawa et al., 1981) or the use of liposomes (Fraley et al., 1982) or calcium-phosphate (Krens et al., 1982) techniques. These chimeric genes should also prove useful as markers in somatic hybridization experiments or as sensitive probes for studying promoter function. Finally, two obvious but significant aspects of the results presented in Fraley et al., 1983, are (*i*) it should now be possible, by using Ti plasmids that have the tumor genes (i.e., tms and tmr loci, Garfinkel et al., 1981) deleted, to obtain kanamycin-resistant transformants that can

be readily and reproducibly regenerated into phenotypically normal plants, and (*ii*) there is no reason to believe that NPTase I and NPTase II are unique in their ability to be expressed in plant cells and it is quite likely that other bacterial, fungal, or mammalian genes, including those whose products could be expected to modify plant properties in a useful manner, could also be successfully engineered and expressed.

Simple coincubation (growing plant cells and bacterial cells together) has been shown to be extremely effective in transforming plant protoplasts and leaf disks, and whole transformed plants have now been regenerated in numerous plant species (see Horsch et al., 1984). Horsch et al., 1984, states as follows:

Morphologically normal plants were regenerated from *Nicotiana plumbaginifolia* cells transformed with an *Agrobacterium tumefaciens* strain containing a tumor-inducing plasmid with a chimeric gene for kanamycin resistance. The presence of the chimeric gene in regenerated plants was demonstrated by Southern hybridization analysis, and its expression in plant tissues was confirmed by the ability of leaf segments to form callus on media containing kanamycin at concentrations that were normally inhibitory. Progeny derived from several transformed plants inherited the foreign gene in a Mendelian manner.

*Agrobacterium tumefaciens*, the causative agent of crown gall disease, is capable of transferring a DNA segment (designated T-DNA), located between specific border sequences, from its tumor-inducing plasmid (Ti plasmid) into the nuclear DNA of infected plant cells (Chilton et al., 1977; Van Larebeke et al., 1974; Chilton et al., 1980; Yadav et al., 1980; Willmitzer et al., 1980). Expression of T-DNA-encoded tumor genes in the transformed cell provides a selectable trait for recognition of those cells in culture; namely, the ability to grow on medium without added phytohormones. Unfortunately, this trait interferes with regeneration of normal fertile transformed plants (Braun, 1956; Braun et al., 1976; Yang et al., 1980).

Recently, Fraley et al, 1983, and others (Herrera-Estrella et al., 1983) have constructed chimeric genes that function as dominant

selectable markers in plant cells, thus making the tumor genes unnecessary for identification of transformants. The chimeric gene of Fraley et al., 1983, and Horsch et al., 1984, contains the coding sequence of the bacterial gene for neomycin phosphotransferase II (NPTII) joined to the 5' and 3' regulatory regions of the nopaline synthase (NOS) gene, which is expressed constitutively in higher plant cells (Tempé et al., 1982). Fraley et al., 1983, has shown that petunia and tobacco cells transformed with this chimeric NOS/NP-TII/NOS gene are readily selected and are highly resistant to kanamycin.

Horsch et al., 1984, now report that kanamycin-resistant plant cells obtained with their vector system regenerate to morphologically normal plants. These plants carry a functional kanamycin-resistance gene and produce viable seeds. Analysis of progeny shows that the chimeric kanamycin-resistance gene is inherited and is expressed as a dominant Mendelian trait.

Horsch et al., 1984, used the previously described pMON120 intermediate vector to introduce the chimeric NOS/NPTII/NOS gene into the *A. tumefaciens* Ti plasmid. The pMON120 plasmid also contains an intact NOS gene as a scorable transformation marker. This NOS fragment includes the nopaline-type T-DNA right border sequence (Zambryski et al., 1982; Yadav et al., 1982). Because this additional border sequence is initially carried on a separate plasmid, Horsch et al., 1984, refers to their system as the split end vector (SEV) system. Figure 6 shows how this system is used. The pMON128 plasmid, pMON120 containing the chimeric kanamycin-resistance gene (Fig. 6B), is introduced by conjugation into *A. tumefaciens* cells, where homologous recombination with a resident octopine-type Ti plasmid, pTiB6S3 (Fig. 6A), occurs. The resultant cointegrate plasmid pTiB6S3::pMON128 (Fig. 6C) contains a hybrid T-DNA in which the nopaline-type right border sequence is positioned between the kanamycin-resistance gene and the tumor genes of the resident Ti plasmid. Use of the nopaline T-DNA border sequence during infection results in the transfer of a short T-DNA segment (Fig. 6E) which contains the kanamycin-resistance gene and an intact NOS gene but does not contain genes for tumor formation or octopine synthase. The short-transfer

transformants can be regenerated to give intact plants as described below.

Transformation of *Nicotiana plumbaginifolia* cells was carried out with the engineered *A. tumefaciens* train containing the chimeric NOS/NPTII/NOS gene by the method of cocultivation (Fraley et al., 1983). Leaf mesophyll protoplasts of *N. plumbaginifolia* were obtained and cultured as described by Pollock et al., 1983. Two-day-old cultures of protoplast-derived cells were inoculated with live *A. tumefaciens* cells ($10^7$ cells per milliliter). After 48 hours of coculture, the plant cells were collected by centrifugation (100*g* for 5 minutes), washed twice, and replated in medium containing carbenicillin (500 µg/ml) to kill the remaining bacteria. On the sixth day after isolation of protoplasts, the procedure described for petunia cells (Fraley et al., 1983) was used to transfer the cultures from liquid to feeder plates (Horsch et al., 1980) of the same medium. After 7 to 10 days on the feeder plates, the colonies were transferred to fresh medium (without feeder cells). After another 7 days, colonies were transferred to a different medium, MS salts (Gibco), B5 vitamins, 3 percent sucrose, carbenicillin (500 µg/ml), kanamycin (100 µg/ml), benzyl adenine (1.0 µg/ml), and naphthalene acetic acid (0.1 µg/ml); *p*H 5.7. After 7 days, the most promising colonies (largest and greenest) were picked from the filter paper substrate and transferred to the same medium at low density, 10 colonies per petri plate (100 by 15 mm).

In the first experiment of Horsch et al., 1984, four kanamycin-resistant colonies were recovered from approximately $8 \times 10^4$ protoplasts cocultivated with pTiB6S3::pMON128. No resistant colonies were found among the same number of untreated control colonies. Southern blot hybridization with an NPTII specific probe showed that all four resistant colonies contained the chimeric gene (data not presented). One of the four colonies was morphogenic and produced a kanamycin-resistant plant, NPK3.

Leaf segments from NPK3 were able to form callus and proliferate new shoots on medium containing kanamycin (100 µg/ml) (Fig. 7A). In contrast, leaf segments from wild-type plants were completely inhibited. Analysis with Southern blot

hybridization showed that the 1.5-kilobase (kb) Eco RI fragment containing the chimeric NOS/NPTII/NOS gene was present in the leaves of NPK3 but not in tissue from wild-type plants (lanes c and d in Fig. 7B).

Twenty-one first-generation progeny plants ($S_1$) from the self-fertilized transformed parent NPK3 were grown to maturity and tested for kanamycin resistance in the leaf callus assay. Fifteen of the 21 were able to form callus on medium with kanamycin (100 µg/ml). Another 80 seedlings (germinated under sterile conditions) were transferred to medium containing kanamycin (100 µg/ml). Of these, 62 grew several times larger and formed callus, whereas 18 ceased growth and did not form callus. Thus the trait was inherited in a Mendelian manner with a 3:1 ratio. The final proof of the correspondence between the presence of the chimeric gene and the antibiotic-resistant phenotype was established by the perfect correlation between inheritance of the chimeric gene and the kanamycin-resistant phenotype in $S_1$ plants (lanes e to n in Fig. 7B).

Three subsequent cocultivation experiments gave high frequencies of transformation, averaging 6 percent of the total colonies or 1.2 percent of the total initial protoplasts plated. The control populations consistently failed to yield any kanamycin-resistant colonies. Most (about 90 percent) of the kanamycin-resistant colonies produced both octopine and nopaline and were nonmorphogenic, as expected for transformants arising when the octopine T-DNA right border was utilized (Fig. 6D). About 10 percent of the colonies were morphogenic, producing shoots that could be excised, rooted, and grown in soil. Of 22 plants examined, 9 were escapes or revertants that showed none of the markers of transformation. The other 13 plants produced nopaline, but not octopine, and were resistant to kanamycin as measured by a leaf callus-induction assay.

The $S_1$ progeny from three of the independently isolated, nopaline-producing and kanamycin-resistant plants (NPK7, NPK9, NPK10) were scored for nopaline content. In each case, the progeny showed normal Mendelian inheritance and expression of the inserted DNA segment: 71 of 105 progeny of NPK7, 39 of 48

progeny of NPK9, and 34 of 44 progeny of NPK10 produced nopaline. In addition, axenically grown seedings from each of the transformants showed similar segregation for ability to form callus on medium containing kanamycin (100 μg/ml). For example, 37 of 51 progeny of NPK10 formed callus in the presence of kanamycin, and all 37 resistant progeny produced nopaline.

Horsch et al., 1984, has shown that (i) the chimeric NOS/NPTII/NOS gene is expressed in *N. plumbaginifolia*, (ii) the regenerated transformed plants are phenotypically normal and fertile, and (iii) normal Mendelian inheritance of an engineered gene can occur in the progeny of transformed plants. The genetic transmission of chimeric antibiotic-resistance genes has now been confirmed for the $S_2$ progeny of the NPK3 plant.

Normal Mendelian inheritance of the chimeric gene has also been demonstrated for petunia plants transformed with pMON120-type vectors. The availability of dominant selectable markers and transformation vectors that permit the regeneration of phenotypically normal plants will greatly facilitate studies of gene expression and regulation in plants.

In mammals, naturally infective retroviruses have been used to construct naturally transforming vectors which insert engineered DNA into the mammalian chromosome, in a manner similar to *Agrobacterium tumefaciens*. This transformation mechanism is considered extremely promising for animal and human gene therapy (see Anderson, 1984).

For an example of mammalian transformation, see U.S. Pat. No. 4,396,601 to Salser et al., which describes a technique in which cells are isolated from a regenerative body member of a mammal or a syngeneic equivalent to provide parent cells. The parent cells are combined with DNA from the parasite and with additional DNA that produces a a selection advantage over the parent cells when the cells are subjected to mitotic inhibition. The modified cells are then introduced into the host in a manner such that the modified cells return to the body member from which the parent cells were obtained. A mitotic inhibitor is then administered to the host to provide a selective advantage for the modified cells over the parent cells, thereby regenerating the modified cells in the host. Further details of this method can be obtained by reference to U.S. Pat. No. 4,396,601.

The method of the invention is generally applicable to the protection of any host from a parasite of that host. As used herein, "host" refers to any organism that can be infected by any parasitic or symbiotic organism. The term "parasite" refers to any organism that obtains substance or means for reproduction from an organism, whether it lives with that organism in a parasitic or symbiotic relationship. The parasite need not be specific for a particular host but may be a parasite of many hosts, such as the caterpillars of numerous moths and bufferflies. Although a preferable parasite for use in this invention is a virus, whether the virus is a DNA or RNA virus, other parasites are also encompassed by this term. Examples of other parasites include bacteria, protozoa, fungi, nematodes, insects, and arachnids.

Since a host is normally higher in the evolutionary scheme than the parasite, the term "host" does not encompass a virus, which resides at the bottom of the evolutionary scheme. However, any higher organism is capable of being infected by a parasite. The invention is readily applicable, for example, to bacteria grown in culture which need protection against infection from bacteriophages. Additionally, plants and other higher organisms, such as mammals, also can be readily protected from viruses using the method of the invention. Both plants and animals can also be protected from higher parasitic hosts, such as insects and protozoans, subject to the restrictions which have already been discussed. Examples of hosts include bacteria, yeasts, fungi (e.g., mushrooms), legumious plants (e.g., soybeans), cereal and forage crops (e.g., corn, wheat, rice and alfalfa), food crops (e.g., tomatoes, potatoes, lettuce, and onions), ornamental plants (e.g., roses, junipers, and orchids), trees (e.g., pine, spruce, and walnut), protozoans, amphibians, reptiles, birds (e.g., chickens and turkeys), and mammals (e.g., cats, dogs, horses, cattle, sheep, goats, pigs, and primates).

Examples of host/parasite systems in which either the host or the parasite is a unicellular organism (the most common situations) can be found in numerous microbiology textbooks and reference manuals, such as CRC Handbook of Microbiology, Condensed Edition, Laskin and Lechevalier (eds.), CRC Press, Cleveland, Ohio, 1974. Other examples of host/parasite systems are given below along with examples of how resistance to the parasite can be given to the host in that system. These examples are not limiting, and many other methods for achieving resistance are possible for each listed system.

1) There are a variety of bacteria important in industrial fermentation processes, such as *Streptococcus lactis, Streptococens cremoris*, and *Lactobacillus species*. During fermentation, infection by various bacteriophages is a common cause of fermentation failure. Bacterial resistance to such bacteriophage infection can be engineered by methods exactly analogous to the methods described above for engineering resistance to the Qβ bacteriophage in *E. coli*.

2) There are hundreds of significant plant RNA viruses, and essentially all crop species are affected by one or more such viruses. Resistance to such viruses can be obtained in a manner closely analogous to Qβ resistance in bacteria, by cloning fragments of the viruses into plant-transforming vectors such as a modified Ti-plasmid and transforming the appropriate plants. Plants transformed by various gene fragments can then be screened for resistance, using established plant breeding techniques. A few relevant viruses include alfalfa mosaic virus, brome mosaic virus, barley yellow dwarf virus, beet yellows virus, cucumber mosaic virus, lettuce necrotic yellows virus, maize chlorotic dwarf virus, pea enation virus, potato viruses S, X, and Y, southern bean mosaic virus, tomato ringspot virus, tobacco ringspot virus, tobacco mosaic virus, tobacco streak virus, turnip yellow mosaic virus, and wound tumor virus.

3) There are certain animal and human pathogens, such as the flu and common cold viruses, which have evolved mechanisms for circumventing the effectiveness of the animal immune system. Where such a virus is a chronic problem, as with flu and colds, parasite-derived resistance will be a powerful tool for conferring immunity to all strains of that pathogen. Resistance can be engineered by cloning fragments of the viral genome, introducing the gene fragments into animal cells in vitro by use of retroviral vectors, testing of varius tranformed cell times to determine which have conferred resistance to infection by the virus, and then using those fragments conferring resistance to create benign non-infectious retrovirus vectors for the purpose of introducing resistance genes into individuals.

4) There are certain retroviruses which attack T-cells (i.e., the human immune system) directly (such as the viruses that produce AIDS), thereby circumventing our natural immune defense mechanism.

Resistance can be engineered as described above, using AIDS genomic fragments, and also using AIDS, or a similar retrovirus, for the construction of a T-cell-specific transforming vector. Transformed T-cells with resistance-conferring fragments of the AIDS genome would have a selective advantage over other susceptible T-cells, becoming the predominant form of T-cell and thereby giving rise to resistant individuals.

5) A wide range of bacteria and fungi that parasitize plants have intimate contact with living host cells and reveal gene-for-gene host parasite relations. Resistance in such cases can be engineered by cloning avirulence alleles from avirulent strains of the parasite and introducing these genes into the relevant host for the purpose of conferring resis-

tance. A few pathogens where this method is relevant include *Puccinia sorghi* infection of corn. Puccinia infections of wheat. *Phytophthora infestans* infection of potato. Ustilago infection of rye. and *Melampsora lini* infection of flax.

6) A wide range of insects parasitize plants. causing severe economic losses. and depend upon a proper balance of juvenile hormone and molting hormone to regulate their development. Therefore. broad-spectrum. insect-derived plant resistance to insects can be engineered by cloning the insect genes responsible for the final steps of the biosynthesis of these hormones and transferring these genes to the plant hosts of interest. using established transformation techniques. Typical genes would code for enzymes controlling the conversion of a precursor into the desired regulatory product (e.g.. hormone). Basically all plant hosts contain the precursors for the synthesis of these hormones; i.e.. farnesol in the case of juvenile hormone and phytosterols in the case of molting hormone. Other useful genes would be those producing other regulatory substances that trigger the production of hormones in parasites. A few insect parasites which could be controlled by this method include flea beetles. wire worms. cutworms. grubs. aphids. leafhoppers. tarnished plant bugs. Colorado potato beetles. cucumber beetles. weevils. cabbage worm. cabbage lopper. leafminers. Hessian fly. grasshopper. tent worm. gypsy moth. tussock moth. army worm. corn ear worm. European corn borer. and Japanese beetle.

7) A wide range of insects parasitize plants and contain neurotransmitters which control essential body functions. Such neurotransmitters are oligopeptides typically only 5–20 amino acids long. In this case insect-derived resistance can be engineered by sequencing the oligopeptide and synthesizing artificial genes homologous to the native insect genes coding for these neurotransmitters. These synthetic genes. when expressed in a plant host. can then disrupt that crucial body function normally regulated by that neurotransmitter of the insect parasite. The insect listed in the previous example would be equally valid as candidates for this method of deriving parasite-derived resistance.

In addition to the above general procedures which can be used for preparing recombinant DNA molecules and transformed unicellular organisms in accordance with the practices of this invention, other known techniques and modifications thereof can be used in carrying out the practice of the invention. In particular. techniques relating to genetic engineering have recently undergone explosive growth and development. Many recent U.S. patents disclose plasmids. genetically engineered microorganisms. and methods of conducting genetic engineering which can be used in the practice of the present invention. For example. U.S. Pat. No. 4.273.875 discloses a plasmid and a process of isolating the same. U.S. Pat. No. 4.304.863 discloses a process for producing bacteria by genetic engineering in which a hybrid plasmid is constructed and used to transform a bacterial host. U.S. Pat. No. 4.419.450 discloses a plasmid useful as a cloning vehicle in recombinant DNA work. U.S. Pat. No. 4.362.867 discloses recombinant cDNA construction methods and hybrid nucleotides produced thereby which are useful in cloning processes. U.S. Pat. No. 4.403.036 discloses genetic reagents for generating plasmids containing multiple copies of DNA segments. U.S. Pat. No. 4.363.877 discloses recombinant DNA transfer vectors. U.S. Pat. No. 4.356.270 discloses a recombinant DNA cloning vehicle and is a particularly useful disclosure for those with limited experience in the area of genetic engineering since it defines many of the terms used in genetic engineering and the basic

processes used therein. U.S. Pat. No. 4,336,336 discloses a fused gene and a method of making the same. U.S. Pat. No. 4,349,629 discloses plasmid vectors and the production and use thereof. U.S. Pat. No. 4,332,901 discloses a cloning vector useful in recombinant DNA. Although some of these patents are directed to the production of a particular gene product that is not within the scope of the present invention, the procedures described therein can easily be modified to the practice of the invention described in this specification by those skilled in the art of genetic engineering.

[All of the patents and other publications cited in this specification are indicative of the level of skill and knowledge of those skilled in the arts to which the present invention pertains. All publications, whether patents or otherwise, referred to previously or later in this specification are herein separately incorporated by reference. Although full incorporation of the individual publications is intended, it is recognized that those of ordinary skill in the art can readily determine from the incorporated publications those sections which are most relevant to the present invention and those sections which could be deleted without loss of understanding.]

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Publications specifically excluded from incorporation by reference into this specification are as follows:

Adams, S.P. et al., *J. Am. Chem. Soc.*, 105:661-663 (1983).

Akiyoski, D. et al., *Proc. Natl. Acad. Sci. USA*, 80:407-411 (1983).

Ausubel, F. et al., *Plant Mol. Newsl.*, 1:26-32 (1980).

Bale, A. et al., *Mutat. Res.*, 59:157-165 (1979).

Barton, K. et al., *Cell*, 32:1033-1043 (1983).

Beck, E. et al., *Gene*, 19:327-336 (1982).

Berg, D. et al., *Proc. Natl. Acad. Sci. USA*, 76:3628-3632 (1975).

Bevan, M. et al., *J. Mol. Appl. Genet.*, 1:539-546 (1982).

Braun, A., *Cancer Res.*, 16:53-56 (1956).

Braun, A. et al., *Proc. Natl. Acad. Sci. USA*, 73:496-500 (1976).

Chilton, M.D. et al., *Cell*, 11:263-271 (1977).

Chilton, M.D. et al., *Proc. Natl. Acad. Sci. USA*, 77:4060-4064 (1980).

Chilton, M.D. et al., *Stadler Genet. Symp.*, 13:39-51 (1981).

Chilton, M.D. et al., *Nature (London)*, 295:432-434 (1982).

Colbere-Garapin, F. et al., *J. Mol. Biol.*, 150:1-14 (1981).

Colman, A. et al., *Eur. J. Biochem.*, 91:303-310 (1978).

Covarrubias, L. et al., *Gene*, 13:25-35 (1981).

Davies, R.W. et al., *Advanced Bacterial Genetics* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), p. 116 (1980).

DeGreve, H. et al., *Plasmid*, 6:235-248 (1981).

Depicker, A. et al., *J. Mol. Appl. Genet.*, 1:561-574 (1982).

Ditta, G. et al., *Proc. Natl. Acad. Sci. USA*, 77:7347-7351 (1980).

Fraley, R. et al., *Current Topics in Microbiology and Immunology*, eds. Hofschneider, P. & Goebel, W. (Springer, New York), 171-192 (1982).

Garfinkel, D. et al., *Cell*, 27:143-155 (1981).

Gelvin, S. et al., *Plasmid*, 6:17-29 (1981).

Goeddel, D. et al., *Nucleic Acids Res.*, 8:4057-4074 (1980).

Hasezawa, S. et al., *Mol. Gen. Genet.*, 182:206-210 (1981).

Herrera-Estrella et al., *EMBO J.* 2:987 (1983).

Hille, J. et al., *Plasmid*, 7:107-118 (1982).

Holsters, M. et al., *Plasmid*, 3:212-230 (1980).

Horsch, R. et al., *In Vitro*, 16:103-108 (1980).

Ish-Horowicz, D. et al., *Nucleic Acids Res.*, 9:2989-2998 (1981).

Jimenez, A. et al., *Nature (London)*, 287:869-871 (1980).

Kerr, A. et al., *Nature (London)*, 265:560-561 (1977).

Klee, H. et al., *Bacteriol*, 150:327-331 (1982).

Leemans, J. et al., *EMBO J.*, 1:147-152 (1982).

Messing, J. et al., *Nucleic Acids Res.*, 9:309-321 (1981).

Messing, J. et al., *Gene*, 19:269-276 (1982).

Murray, N.E. et al., *J. Mol. Biol.*, 132:493-505 (1979).

Nagao, R. et al., *DNA*, 1:1-9 (1981).

Oka, A. et al., *J. Mol. Biol.*, 147:217-226 (1981).

Otten, L. et al., *Biochim. Biophys. Acta*, 527:497-500 (1978).

Otten, L. et al., *Mol. Gen. Genet.*, 183:209-213 (1981).

Pollock, K. et al., *Plant Cell Rep.* 2:36 (1983).

Rogers, S.G. et al., *Gene*, 11:187-195 (1980).

Southern, E., *J. Mol. Biol.*, 98:503-517 (1975).

Southern, P. et al., *J. Mol. Appl. Genet.*, 1:327-341 (1982).

Spano, L. et al., *Pflanzenphysiol.*, 106:87-92 (1982).

Tempé, J. et al., *Molecular Biology of Plant Tumors*, eds. Kahl, G. & Schell, J. (Academic, New York), 427-449 (1982).

Van Larebeke, N. et al., *Nature (London)*, 252:169-170 (1974).

White, F. et al., *Proc. Natl. Acad. Sci. USA*, 79:3193-3198 (1982).

Willmitzer, L. et al., *Nature (London)*, 287:359-361 (1980).

Willmitzer, L. et al., *EMBO J.*, 1:139-146 (1982).

Wullems, G. et al., *Cell*, 24:719-727 (1981a).

Wullems, G. et al., *Proc. Natl. Acad. Sci. USA*, 78:4344-4348 (1981b).

Yadav, N. et al., *Nature (London)*, 287:458-461 (1980).
Yadav, N. et al., *Proc. Natl. Acad. Sci. USA*, 79:6322-6326 (1982).
Yang, F. et al., *Mol. Gen. Genet.*, 177:707-714 (1980).
Zambryski, P. et al., *J. Mol. Appl. Genet.*, 1:361-370 (1982).

In addition to the method of producing resistance to a parasite described above in detail, this invention also encompasses hosts produced by the process of the invention as well as recombinant vectors and other products of genetic engineering useful in the practice of the invention.

The invention now being generally described, the same will be better understood by reference to certain specific examples which are included herein for purposes of illustration only and are not intended to be limiting of the invention or any embodiment thereof, unless so specified.

## EXAMPLE

The feasibility of the concept outlined above was proven with experiments using the bacteriophage Qβ and its host, *E. coli*. Using cDNA clones of Qβ (Qβ is an RNA phage), plasmids were first constructed or obtained which would express part of the Qβ cDNA in *E. coli* and confer resistance.

Coat Protein

The plasmid used for production of the coat protein was pGL101 obtained from R. B. Winter (*Cell* 33, 877). This plasmid expresses the coat protein under lac operator control, so its expression can be induced by IPTG (though there is also a constitutive expression). This plasmid as well as the others described below contain the gene encoding $amp^r$.

Negative Strand

A plasmid was constructed that inserted the 0.9 Kb HpaII fragment of Qβ cDNA into pUR222 plasmid at the AccI site. This fragment extends from positions 2547 to 3473 in Qβ (FIGURE) and includes translational sequences of the replicase gene. These sequences also contain the M-binding site of the replicase. In the "sense" orientation of this fragment, a fusion product between β-galactosidase protein and the replicase fragment is formed. In the antisense (reverse) ligation of this fragment, an RNA complementary to the Qβ RNA sequence is formed. Both constructions were made.

Testing for Resistance

The strain GM1 (provided by R. W. Webster) was transformed with pUR222 or one of the test plasmids described above. These strains were grown up, made competent, incubated with Qβ and then plated out in soft agar. Plaque numbers and sizes were assessed to determine if resistance was taking place.

In an initial experiment to test the coat protein, GM1+pUR222 and GM1+pGL101 were grown in 10 mls L-broth

containing ampicillin in IPTG. At stationary phase the cultures were pelleted and resuspended in 4 ml 50 mM $YCaCl_2$. A small portion, 0.1 ml, of this plating culture was incubated 60' with $10^7$ pfu of Qβ. This was then plated on YT-AMP plates in 3 ml soft agar with IPIG. The results were that the GM1+pUR222 plates had thousands of plaques which soon (24 hrs) engulfed the plate; the GM1+pGL101 plate at first showed no plaques but later developed many very small plaques.

To check the possibility that the GM1 strain+pGL101 resistance was due to loss of the F' element, the strains were subsequently grown on minimal medium lacking proline to maintain selection for the F'. The same protocol as above was then repeated, including strains of GM1 with the HpaII (sense) and HpaII (antisense) bearing plasmids. The results are presented in Table 1. Both the coat protein and the HpaII (antisense) plasmids could confer resistance to Qβ infection. This experiment was repeated twice with essentially the same results. After continued passage, however, the plasmid bearing Qβ cDNA sequences rearranged or were lost. Additionally, the pGL101 was tested at higher titer ($10^{11}$pfu); it still conferred resistance. Coat-conferred resistance from the RNA phages f1 and f2 were tested. GM1 with pGL101 was resistant to f2 but not f1 as might be expected considering their nodes of infection.

TABLE 1

| Strain + Plasmid | | Inducer | # of Plaques | Size |
|---|---|---|---|---|
| GM1 | — | + | 300 | normal |
| | puR222 | – | 360 | normal |
| | puR222 | + | 348 | normal |
| | Hpa (antisense) | – | 247 | normal |
| | Hpa (antisense) | + | 263 | small |
| | Hpa (sense) | – | 224 | normal |
| | Hpa (sense) | + | 234 | normal |
| | pGL101 | – | 176 | very small |
| | pGL101 | + | 101 | very very small |

Replicase Binding Domain

In an experiment similar to those described above, a model system involving *E. Coli* and its viral pathogen Qβ was utilized. A pBR322 plasmid containing a cDNA clone from the 3' end of the Qβ genome was obtained from Martin Billeter at the University of Zurich. The gene segment of this plasmid encodes all of the replicase gene of Qβ. Various DNA constructions were made using this source of a gene encoding the Qβ viral replicase. The constructs were made by annealing a 5' Sau3a site in the replicase coding region to a BamHI site in the lacZ gene on the pUC18 plasmid. The pUC18 plasmid is commercially available from Bethesda Research Laboratories. The restriction enzymes used in producing the fragments as well as various other enzyme used in the genetic engineering steps described herein are also commerically available.

This process created a gene, when in-frame, encoding a few amino-terminal amino acids of lacZ followed by a replicase protein domain terminating with a NarI site beyond the transcriptional termination site of the replicase enzyme. Since the restriction enzyme Sau3a recognizes a large number (approximately 8) of cleavage sites in the replicase gene, a number of different DNA constructs were produced. The plasmids produced in this manner resulted in the production of the replicase domain being under lacZ promoter control. Some of the Sau3a sites in the replicase gene produced an out-of-frame fusion of replicase.

The vector constructions so made were transformed into *E. Coli* JM103, a commerically available strain. The size of

the replicase gene fragment in each construction was determined, and the susceptibility of each transformant to Qβ infection was tested.

Basically, there were two classes of fusions. One class contained approximately 1,000 base pairs of the 3' end of the replicase (4 out of 5 should be in-frame) and one class contained approximately 500 base pairs of the 3' end (2 of 3 would be out-of-frame). The 1,000-BP class was represented by clones #7, #10 and #11, while the 500-BP class was represented by #4, #5, and #8 (see Figure). The colonies containing these constructions were grown in L-broth and then in M-9 media to select for male *E. coli*. Both of these media are commercially available and are described in Maniatis et al, *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratories, Cold Spring Harbor, N.Y. (1982). A plating culture was then made either with or without IPTG-induction (IPTG is isopropyl thiogalactoside). It was initially suspected that induction of lacZ expression with IPTG would be needed to produce enough replicase protein fragment in order to see protection From infection. However, as shown below, this did not prove to be necessary.

The results of the comparative tests are seen in Table 1 below. Plasmid pUC9 is JM103 with the parental plasmid which served as a negative control. The reference 2290 is to the original whole-replicase-containing plasmid in JM103. All of the replicase fusion products produced a 10-fold or more protection against infection except construction #8. This construction had the smallest portion of the replicase gene and may not be in-frame. All of the strains were about equally infectable as determined by infection with a different pilus-specific phase, f2.

TABLE I

| | | No. of plaques/plate | | |
|---|---|---|---|---|
| | Strain with Plasmid | -IPTG | +IPTG | f2 |
| | PUC9 | 240 | 2400 | 172 |
| | 2290 | 294 | 600 | 116 |
| | #7 | 250 | 87 | 332 |
| 1000 bp | #10 | 166 | 53 | 348 |
| | #11 | 264 | 16 | 279 |
| | #4 | 243 | 36 | 277 |
| 500 bp | #5 | 208 | 144 | 266 |
| | #8 | 240 | 1342 | 215 |

The results set forth above indicate that bacteria (and by inference other hosts) can be protected against infection with an RNA virus by inserting a gene controlling production of an inoperative fragment of a viral RNA replicase enzyme into a host organism.

The invention now being fully described, it will be apparent to one of ordinary skill in the art that many changes and modifications can be made thereto without departing from the spirit or scope of the invention as set forth herein.

REFERENCES CITED

Ames, B. N. (1983). *Science* 221:1256–1264.
Anderson, W. F. (1984).*Science* 226:410–409.
Bajaj, Y. P. S. (1982). In: Reinert, J. and Bajaj, Y. P. S. (eds.), *Plant Cell, Tissue and Organ Culture*. Springer-Verlag, New York, pp. 467–496.
Baserga, R., C. Crose, G. Rovera, Eds. (1980). Introduction of macromolecules into viable mammalian cells. In: *Sinstar Symposium Series VI*. A. R. Liss Inc. New York.
Bernardi, A. and Spahr, P. (1972). *Proc. Nat. Acad. Sci.* 69:3033.
Bowers, W. S. (1980). In: *Insect Biology in the Future* p. 613. New York, Academic Press.
Chang, S. and Cohen, S. N. (1979). *Mol. Gen. Genet.* 168:111–115.
Cocking, E. C. (1972). *Ann. rev. Plant Physiol.* 23:29–50.
Diacumakos, E. G. (1973). In: Prescott, D. M. (ed.), *Methods in Cell Biology*. Academic Press, New York, pp. 287–311.
Dityatkin, S. Y., Lisovskaya, K. V., Panzhava, N. N., Iliashenko, B. N. (1972). *Biochimica et Biophysica Acta* 281:319–323.
Dorssers, L., Meer, J. van der, Kammen, A. van, and Zabel, P. (1983). *Viroloqy* 125:155–174.
Fairfield, A. S., S. R. Meshnick, and J. W. Eaton. (1983). *Science* 221:764–766.
Flashman, S. F. (1978). *Mol. Gen. Genet.* 166:61–73
Flor, H. H. (1971). *Ann. Rev. Phytopathol.* 9:27S-296.
Fraley, R. T., et al. (1983). *Proc. Natl. Acad. Sci.* 80:4803–4807.
Gibbs, A. (1969). *Adv. Virus Res.* 14:263–327.
Graessmann, M. and Graessmann, A. (1983). *Methods in Enzymology* 101:482–492.
Graham, F. L. and van der Eb, A. J. (1973). *Virology* 52:456.
Hall, T. C., W. A. Miller, and J. J. Bujarski. (1982). In: *Advances in Plant Pathology*. P. 179. (Vol. 1) Academic Press, New York.
Hamilton, R. I (1980). In: *Viruses. Plant Disease: An Advanced Treatise*. P. 279. (Vol. 5). Academic Press, NY.
Held, G. A., L. A. Bulla, E. Ferari, J. Hoch, A. I. Aronson, and S. A. Minnich (1982). *Prov. Natl. Acad. Sci.* 79:6065–6069.
Horsch, R. B., R. T. Fraley, S. G. Rogers, P. R. Sanders, A. Lloyd, N. Hoffmann (1984). *Science* 223:496–498.
Izant, J. and Weintraub, H. (1984). *Cell* 36:1007–1015.
Kamen, G. and P. Argos (1984). *Nucleic Acids Res.* 12:7269
Kamen, R. (1970). *Nature* 228:527–553.
Karnik, S. and Billeter, M. A. (1983). *EMBO* 2:1521.
Krens, F. A., Molendijk, L., Wullems, G. J., and Schilperoort, R. A. (1982). *Nature* 296:72.
Mandel, M. and Higa, A. (1970). *J. Mol. Biol.* 53:159–162.
Marcus, P. I. and Carrier, D. H. (1967). *J. Virology* 1:334.
Marcus, P. I. and Zuckerbraun, H. L. (1969). In: *The Biology of Large RNA Viruses*. P. 455. Acad. Press, New York.
Marcus, P. I. and Zuckerbraun, H. L. (1970). *Ann N.Y. Acad. Sci.* 173:185–198.
Meyer, F., H. Weber, and C. Weissmann. (1976). *Experientia* 32:804.
Meyer, F., Weber, H. and Weissman, C. (1981). *J. Mol. Biol.* 153:631–660.
Mizuno, T., Chou, M. and Inouye, M. (1984). *Proc. Natl. Acad. Sci. USA* 81:1966–1970.
Mouches, C., Candresse, T. and Bove, J. M. (1984). *Virology* 134:78–90.

Ocho, M., Nakai, S., Tasaka, K., Watanabe, S., and Oda, T. (1981). *Acta Med. Okayama* 35 (5):381–384.
Potrykus, I. (1973). *Z. Pflanzenphysiol.* 70:364–366.
Power, J. B. and Cocking, E. C. (1977). In: Reinhert, J., and Bajaj Y. P. S. (eds.) *Plant Cell, Tissue and Organ Culture.* Springer-Verlag, New York, pp. 497–505.
Purres, R. D. (1981). *Academic Press*, New York, 146 p.
Roberts, T. M., Kacich, R. and Ptashne, M. (1979). *Proc. Natl. Acad. Sci. USA* 76:760–764.
Silverman, P. M., Rosenthal, S., Mobach, H., and Valentine, R. C. (1968). *Virology* 36:142.
Staskawicz, A. J., D. Dahlbeck, and N. Keen (1984). *Proc. Natl. Acad. Sci. USA* 81:6024.
Uchida, T., Yamaizumi, M., Makada, E., Okada, Y. (1980). In: *Introduction of macromolecules into viable mammalian cells.* Windsor Symposium Series VI, A. R. Liss Inc., New York, pp. 169–185.
Uchimiya, H., Ohgawara, T., and Harada, H. (1982). In: Fujiwara A. (ed.), *Proc. 5th Intl. Cong. Plant Tissue and Cell Culture,* Jap. Assoc. for Plant Tissue Culture. Tokyo. pp. 507–508.
Van der Plank, J. E. (1978). *Genetic and Molecular Basis of Plant Pathogenesis.* Springer-Verlag, NY. pp. 167.
Wigler, M. , Sweet, R., Sim, G. K. Wold, B., Pellicer , A., Lacy, E., Maniatis, T., Silverstein, S., and Axel, R. (1979). *Cell* 16:777.
Winter, R. B. and Gold, L. (1983). *Cell* 33:877–855.
Yamamoto, F., Furusawa, M., Furusawa, I., and Obinata, M. (1982). *Exp. Cell Res.* 142:79–84.
Zinder, N. D. (ed) (1975). *RNA Phages.* Cold Springs Harbor Laboratory, NY pp. 428.
Barton, K. A., and Brill, W. J. (1983). Science 219:671–676.
Illmensee, W. (ed.) (1984). Long term Goals in Agricultural Plant Improvement. Genetic Manipulation-Impact on Man and Society. Cambridge Univ. Press.
Day, P. R., Barrett, J. A., and Wolfe, M. S. (1983) Evolution of host-parasite interaction. In: Genetic Engineering of Plants: An Agricultural Perspective. Kosuge, P., Meredith, C. P., and Hollaender, A., eds. Plenum Press, New York.

What is claimed as new and desired to be secured by Letters Patent of the United States is:

1. A method of making a host cells resistant to a virus for the host, comprising:

a) isolating DNA coding for a gene, or fragment thereof of said virus;

b) operably linking said DNA, or a fragment thereof, within an expression vector;

c) transforming said host cells with said expression vector;

d) growing said transformed cells in the presence of said virus, wherein said DNA, or fragment thereof, is expressed as a gene product, and wherein said gene product disrupts an essential activity of said virus; and

e) identifying said transformed host cells which are resistant to infection by said virus by selecting transformed host cells which survive or resist infection by said virus.

2. The method of claim 1, wherein said host cells are bacteria cells, plant cells or plant tissue.

3. A method of making plant cells or plant tissue resistant to infection by one or more virus, comprising:

a) isolating DNA coding for a gene, or fragment thereof, of a virus;

b) operably linking said DNA, or a fragment thereof, within an expression vector;

c) transforming said plant cells or plant tissue with said expression vector;

d) growing said transformed plant cells or plant tissue in the presence of said virus, wherein said DNA, or fragment thereof, is expressed as a gene product, and wherein said gene product disrupts an essential activity of said virus; and

e) identifying said transformed plant cells or plant tissue which are resistant to infection by said virus by selecting said transformed plant cells or plant tissue which survive or resist infection by said virus.

4. The method of claim 3, wherein said DNA or fragment thereof is expressed in the sense direction.

5. The method of claim 3, wherein said DNA or fragment thereof is expressed in the antisense direction.

6. The method of claim 3, wherein said gene or fragment thereof is a replicase gene or fragment thereof.

7. The method of claim 6, wherein said gene or fragment thereof contains a binding site for a replicase enzyme.

8. The method of claim 3, wherein said plant cells or plant tissue are dicotyledonous.

9. The method of claim 3, wherein said expression vector is an *Agrobacterium tumefaciens* plasmid.

10. The method of claim 3, wherein said virus is selected from the group consisting of alfalfa mosaic virus, brome mosaic virus, barley yellow dwarf virus, beet yellows virus, cucumber mosaic virus, lettuce necrotic yellow virus, maize chlorotic dwarf virus, pea enation virus, potato virus S, potato virus X, potato virus Y, southern bean mosaic virus, tomato ringspot virus, tobacco ringspot virus, tobacco mosaic virus, tobacco streak virus, turnip yellow mosaic virus and wound tumor virus.

11. The method of claim 10, wherein said virus is selected from the group consisting of tobacco mosaic virus, cucumber mosaic virus, alfalfa mosaic virus and tobacco ring spot virus.

12. The method of claim 3, wherein said plant cells or plant tissue are tomato cells or tissue.

13. The method of claim 3, wherein said plant cells or plant tissue are cotton cells or tissue.

14. The method of claim 10, wherein said plant cells or plant tissue are potato cells or tissue and said virus is potato virus X.

15. The method of claim 10, wherein said plant cells or plant tissue are potato cells or tissue and said virus is potato virus Y.

16. The method of claim 3, wherein said gene or fragment thereof is a coat protein gene or a fragment thereof.

17. The method of claim 8, wherein said expression vector is an *Agrobacterium tumefaciens* plasmid.

18. The method of claim 17, wherein said virus is selected from the group consisting of alfalfa mosaic virus, brome mosaic virus, barley yellow dwarf virus, beet yellows virus, cucumber mosaic virus, lettuce necrotic yellow virus, maize chlorotic dwarf virus, pea enation virus, potato virus S, potato virus X, potato virus Y, southern bean mosaic virus, tomato ring spot virus, tobacco ring spot virus, tobacco mosaic virus, tobacco streak virus, turnip yellow mosaic virus and wound tumor virus.

19. The method of claim 18, wherein said virus is selected from the group consisting of tobacco mosaic virus, cucumber mosaic virus, alfalfa mosaic virus and tobacco ring spot virus.

20. The method of claim 8, wherein said plant cells or plant tissue are tomato, cotton or potato cells or tissue.

21. The method of claim 3, wherein said virus is an RNA virus.

* * * * *